The Effect of Pollinators, Herbivores and Predators on Floral Trait Evolution

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Anina Catharina Knauer

aus

Zürich ZH

Promotionskommission

Prof. Dr. Florian P Schiestl (Vorsitz und Leitung der Dissertation)

Prof. Dr. Marta B Manser

Prof. Dr. Christian Parisod

Zürich, 2018

TABLE OF CONTENT

ZUSAMMENFASSUNG

Die Interaktion von Pflanzen mit Tieren kann die Blütenevolution und die Diversifizierung der Pflanzen massgeblich beeinflussen. Bestimmte Interaktionen werden jedoch vom Kontext, in dem sie stattfinden, beeinflusst, denn Tiere passen ihr Verhalten und ihre Präferenzen für Blütenmerkmale der Umwelt an. Ausserdem kann die Selektion, welche durch bestimmte Interaktionen ausgeübt wird, von der Korrelation zwischen Pflanzenmerkmalen sowie der Gegenwart anderer Interaktionen abhängen. Diese Faktoren können komplexe Selektionsmuster wie widersprüchliche, korrelierte und nicht-additive Selektion verursachen. Für die Vorhersage der Blütenevolution ist es somit unabdingbar, zu verstehen, wie Pflanzen-Tier-Interaktionen durch den Kontext, in dem sie stattfinden, beeinflusst werden. Allerdings haben bisher die meisten Studien untersucht, wie sich paarweise Interaktionen auf die Selektion und lokale Anpassung von Blüten auswirkt. Somit war das Ziel dieser Arbeit, zum Verständnis der Kontextabhängigkeit von Pflanzen-Tier-Interaktionen und dessen Konsequenzen für die Blütenevolution beizutragen.

In *Kapitel I* untersuchten wir, wie sich die Korrelation zwischen Blütenmerkmalen auf die Präferenz der Bestäuber auswirkt. Da Bestäuber Blüten besuchen um Nahrung (z.B. Nektar) aufzunehmen, sollten sie eine Präferenz für “ehrliche Blütensignale“ haben, welche die vorhandene Nahrungsmenge anzeigen. Wir untersuchten das Vorkommen ehrlicher Signale in *Brassica rapa* (Rübsamen) und die Bedeutung solcher Signale für die Anlockung des generalisierten Bestäubers *Bombus terrestris* (Erdhummel). In unseren Messungen waren zwei Signale mit der Nektarmenge assoziiert: die Kronengrösse und der Duftstoff Phenylacetaldehyd. Tatsächlich hatte diese Assoziation einen Einfluss auf das Verhalten der Bestäuber. Durch den Besuch von Rübsamenblüten, entwickelten die Hummeln eine Präferenz

für den Duftstoff Phenylacetaldehyd im Vergleich zu anderen Duftstoffen. Liessen wir die Hummeln auf Papierblüten mit synthetischen Duftstoffen Nektar sammeln, entwickelten sie eine Präferenz für denjenigen Duftstoff, der jeweils die Nektarqualität ehrlich anzeigte. Diese Ergebnisse demonstrieren, dass die Assoziation eines Blütensignals mit dem Nektar die Attraktivität des Signals massgeblich beeinflussen kann.

Im *Kapitel II* untersuchten wir den Einfluss des Bestäuberverhaltens und der Nährstoffverfügbarkeit im Boden auf die Aufrechterhaltung von ehrlichen Blütensignalen. Da die Bestäuber eine Präferenz für ehrliche Blütensignale ausbilden, sollten in Populationen mit ehrlichen Signalen Pflanzen mit geringer Nektarmenge vom Betrug mit hohen Signalen profitieren. Dennoch gibt *B. rapa* ehrliche Signale ab, der Mechanismus, welcher diese Assoziation aufrechterhält, ist jedoch noch unbekannt. Wir konnten aufzeigen, dass die Blütenbesuchszeit der Hummeln mit der Nektarmenge der Blüten korreliert und die Anzahl Samen, welche die besuchten Blüten entwickelten, erhöht. Ausserdem korrelierten die ehrlichen Blütensignale mit der maximalen Anzahl Samen, welche die Blüten nach Handbestäubung ausbildeten, denn beide Merkmale waren durch den Nährstoffgehalt des Bodens limitiert. Diese Ergebnisse implizieren, dass Individuen mit geringer Nähstoffverfügbarkeit einerseits nur geringe Signale aussenden können und andererseits auf Grund ihrer limitierten Kapazität zur Samenproduktion weniger von grossen Nektarmengen profitieren. Somit werden in *B. rapa* die ehrlichen Signale durch einen Signal-abhängigen differenziellen Nutzen der Nektarproduktion aufrechterhalten, welcher durch eine Kombination von bestäubervermittelter Selektion und Ressourcenlimitierung verursacht wird.

Im *Kapitel III* untersuchten wir die Auswirkung eines bestäubenden Herbivoren auf die Interaktion der Pflanze mit einem Bestäuber. Durch ihre Präferenzen für

Blütensignale können sowohl Bestäuber als auch Herbivoren Selektion auf Pflanzenmerkmale ausüben. Jedoch ist die durch Nützlinge und Schädlinge vermittelte Selektion nicht immer unabhängig voneinander. Die Erdhummeln (*B. terrestris*) und der Schmetterling Grosser Kohlweissling (*Pieris brassicae*) sind beide Bestäuber von *B. rapa*, allerdings ist *B. rapa* auch eine Wirtspflanze der Kohlweissling-Raupen. In unseren Experimenten zeigten sowohl die Hummeln als auch die Schmetterlinge eine Vorliebe für grosse Blüten und eine starke Emission des Duftstoffes Phenylacetaldehyd. Ausserdem lockten die gleichen zwei Signale die Kohlweissling-Weibchen zur Eiablage an. Während die Schmetterlinge in der Abwesenheit der Bienen einen positiven Nettoeffekt auf die Pflanzenfitness hatten, beeinflussten sie die Pflanzenfitness negativ, sobald die Hummeln präsent waren. Insgesamt zeigen diese Ergebnisse den Konflikt auf, welcher zwischen der Anlockung von Nützlingen und der Vermeidung von Schädlingen existiert und widersprüchliche, korrelierte und nicht-additive Selektion verursachte.

In *Kapitel IV* untersuchten wir den Effekt eines generalisierten Räubers auf die Blütenevolution und die Interaktion der Pflanze mit Bestäubern und Herbivoren. Auf Blüten lauernde Räuber können den Pflanzen schaden, indem sie Bestäuber jagen, aber auch nützlich sein, wenn sie Schädlinge fressen. Unsere Feldexperimente mit dem Brillenschötchen *Biscutella laevigata* zeigten einen Konflikt zwischen der Anlockung von Bestäubern und der Vermeidung derer Frassfeinde auf. Denn die Krabbenspinne *Thomisus onustus* reduzierte die Bestäuberbesuche der Blüten, hatte aber die gleiche Vorliebe für den Duftstoff β-Ocimen wie die Bienen. In mit Herbivoren infizierten Pflanzen frassen die Krabbenspinnen jedoch einen Grossteil der Schädlinge und erhöhten damit die Fitness der Pflanzen. Mit Schädlingen befallene Pflanzen hatten ausserdem eine induzierte Emission von β-Ocimen, wobei die Induzierbarkeit in Pflanzenpopulationen, in denen Krabbenspinnen vorkamen,

stärker war als in Populationen ohne Krabbenspinnen. Die Krabbenspinnen bevorzugten überdies Pflanzen von Spinnen-assozierten Populationen, jedoch nur nach einer Infizierung mit Herbivoren. Diese Ergebnisse suggerieren eine lokale Anpassung der Pflanzen an die Gegenwart der Spinnen. Dieses Kapitel veranschaulicht kontextabhängige Selektion auf Blütenmerkmale, welche durch einzelne Pflanzen-Tier-Interaktionen vermittelt wird und deckt zusätzlich den bisher kaum beachteten Effekt von Krabbenspinnen auf die Blütenevolution auf.

Insgesamt demonstriert diese Arbeit die Komplexität der durch Pflanzen-Tier-Interaktionen ausgeübten Selektion auf Blütenmerkmale. In unserer Studie wurde die Evolution der Blüten durch die Korrelation unterschiedlicher Merkmale, durch die Artzusammensetzung der interagierenden Tiere sowie durch abiotische Umweltfaktoren beeinflusst und war somit stark kontextabhängig. Daraus schlussfolgern wir, dass die Auswirkung bestimmter Pflanzen-Tier-Interaktionen auf die Blütenevolution von der räumlichen und zeitlichen Variabilität dieser Faktoren abhängt. Es kann somit davon ausgegangen werden, dass Biodiversitätsverlust, Klimaerwärmung und Nährstoffanreicherung der Böden die Evolution von tierbestäubten Blüten stark verändern wird.

SYNOPSIS

Plant-animal interactions can be key drivers of flower evolution and plant diversification. Specific plant-animal interactions can be affected, however, by the context in which they take place. Animals can adapt their behavior and their preferences for floral traits to different environmental situations. Further, selection imposed by specific interactions can depend on the correlation between plant traits and the presence of other interactions. These factors can cause complex selection patterns on floral traits, as conflicting, correlational and nonadditive selection. Thus, to understand the context-dependence of plant-animal interactions is essential for predicting flower evolution. However, up to date most studies focused on the investigation of pairwise interactions and their effect on the selection and local adaptation of flowers. Thus, the aim of this thesis was to contribute to our understanding of the context-dependence of plant-animal interactions and its consequences for flower-evolution.

In *chapter I* we studied how the correlation between floral traits affects pollinator preferences. As pollinators visit flowers for rewards, they should have a preference for floral signals that indicate reward status ("honest signals"). We investigated honest signalling in *Brassica rapa* and its relevance for the attraction of a generalized pollinator, the bumble bee *Bombus terrestris.* Two floral signals were correlated to reward amounts in *B. rapa*: corolla size and the floral scent compound phenylacetaldehyde. This association indeed affected pollinator behavior. After foraging on *B. rapa*, bumble bees developed a preference for phenylacetaldehyde over dishonest scent compounds. Similarly, when foraging on artificial flowers scented with synthetic volatiles, bumble bees developed a preference for those specific compounds that honestly indicated reward status. These results show that

the association of floral signals with rewards can play a key role in their attractiveness to pollinators.

In *chapter II* we studied the effect of pollinator behavior and nutrient availability in soils on the maintenance of honest floral signalling. As pollinators develop preferences for honest floral signals, in honest signalling plant populations plants with low rewards should profit from cheating by emitting high signals. Nevertheless *B. rapa* emits honest floral signals, the mechanism maintaining the association is still unknown however. We found that flower visitation time by bees was correlated with nectar amount and increased the number of seeds that visited flowers developed. Further, honest floral signals were correlated with the maximal number of seeds that flowers could develop after hand-pollination. Indeed, both traits were limited by nutrients in soil. Together, these results imply that individuals with low nutrient availability in flowers can only produce low values of honest floral signals and at the same time profit less from high nectar amounts than individuals with high signals due to their limited capacities to produce seeds. In *B. rapa*, honest signalling is thus maintained by signal-associated differential benefits of nectar production caused by a combination of pollinator-mediated selection and resource limitation.

In *chapter III* we investigated the effect of a pollinating herbivore on a plant-pollinator interaction. Through their preferences for floral cues, pollinators, but also herbivores, can mediate selection on a variety of plant traits. Selection by mutualists and antagonists may not be independent from each other, however. Bumble bees (*B. terrestris*) and cabbage butterflies (*Pieris brassicae*) are both pollinators of *B. rapa*, but cabbage butterflies also use *B. rapa* as a host plant for their caterpillars. When visiting flowers for rewards, both bumble bees and cabbage butterflies showed a preference for large corollas and strong emission of the floral scent compound phenylacetaldehyde. Additionally, oviposition by butterflies was associated with the

same two floral signals. Further, while cabbage butterflies had a positive net effect on plant fitness in the absence of other pollinators, they affected plant fitness negatively when bumble bees were present. These results demonstrate a conflict between the attraction of mutualists and the avoidance of antagonists which caused conflicting, correlational and non-additive selection

In *chapter IV* we studied the effect of a generalist predator on floral trait evolution and on the plant's interaction with pollinators and herbivores. Flower-dwelling predators can harm plants by hunting pollinators, but also benefit them by feeding on herbivores. We found that the buckler mustard, *Biscutella laevigata*, experienced a conflict between pollinator- and predator attraction. The crab spider *Thomisus onustus* reduced bee visits to flowers, but shared a preference with bees for the floral volatile β-ocimene. In florivore-infested plants, however, crab spiders largely fed on florivores increasing plant fitness. Plants infested with florivores showed an induced emission of β-ocimene, which was stronger in plant populations where crab spiders were present than where they were absent. Crab spiders also preferred plants from populations with spiders, but only after florivore infestation, suggesting plants are locally adapted to the presence of crab spiders. This chapter demonstrates context-dependence of the selection on floral traits imposed by individual plant-animal interactions and discloses the rarely considered relevance of crab spiders for floral signal evolution.

Overall, this thesis demonstrates the complexity of the selection on floral traits imposed by plant-animal interactions. In our study the evolution of floral traits was affected by the association between different floral traits, the species composition of interacting organisms as well as abiotic environmental factors and thus strongly context-dependent. We conclude that the impact of certain plant-animal interactions on floral trait evolution depends on the spatial and temporal variability of these

factors. Thus we expect that biodiversity loss, global warming and nutrient accumulation in soils will strongly influence the evolution of animal-pollinated flowers.

GENERAL INTRODUCTION

EVOLUTION BY NATURAL SELECTION

Where does the spectacular diversity of living organisms on this planet come from?

The first big breakthrough in answering this central question of evolutionary biology had been made in 1859 when Charles Darwin first published *On the Origin of Species by Means of Natural Selection*. In his book, he presented comprehensive evidence that species change through time and are derived from common ancestors. Since then, tremendous evidence for evolution accumulated from fossils and phylogenies as well as micro- and experimental evolution. Further, to support his theory, Darwin also presented a process causing the changes over time – natural selection. This term summarizes four postulates: 1) Individuals within populations are variable; 2) The variation is, at least partially, heritable; 3) In every generation some individuals are more successful at surviving and reproducing than others; 4) The fitness (survival and reproduction) of individuals is associated with the variation among individuals. Individuals with the most favourable traits are better at surviving and reproducing – they are naturally selected. Darwin summarized his idea – the theory of evolution by natural selection - as following:

"*These laws, taken in the largest sense, being Growth with reproduction; Inheritance which is almost implied by reproduction; Variability from the indirect and direct action of the conditions of life, and from use and disuse; a Ratio of Increase so high as to lead to a Struggle for Life, and as a consequence to Natural Selection, entailing Divergence of Character and the Extinction of less improved forms. Thus, from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows. There is grandeur in this view of life, with its several powers, having been originally*

breathed into a few forms or into one; and that, whilst this planet has gone circling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved." (Darwin 1859)

Since then, many studies tested and confirmed the four postulates of natural selection (e. g. Gervasi and Schiestl 2017; Zu et al. 2016; Grant 1999). Evolutionary change can thus be predicted by measuring the strength of selection and heritability. One standardized method to measure selection is the selection gradient, which is calculated as the slope of the regression between the relative fitness (absolute fitness divided by the population mean) and the standardized traits (Lande and Arnold 1983)(Figure 1). Selection can be imposed by abiotic and biotic factors and may vary strongly between geographic regions. Different populations within a species may thus experience different selection pressures and evolve divergently. Such divergent evolution causes local adaptation of populations and can occasionally result in speciation (Leimu and Fischer 2008; Kawecki and Ebert 2004). For example, dark skin color in ectotherms is an adaptation to cold regions, where dark individuals profit from low skin reflectance by improved warming up in the sun (Clusella Trullas et al. 2007). Further, in biotic interactions the interaction partners normally impose selection on each other bidirectionally. For example, in predator-prey interactions the prey is selected for traits that improve defense or escape from predators, while predators themselves are selected to overcome these prey adaptations (Brodie 1999).

PLANT-ANIMAL INTERACTIONS

Because plants are the primary producers on Earth and provide the food for the second trophic level, they built the basis for the radiation of animals. Throughout animal evolution, exploitation of various plant resources has emerged in primary consumers: While some animals can consume whole plants, most rely on specific resources as shoot, leaves, roots, flowers, nectar or pollen (Price 2002). Because of this specificity, most plants interact with various animal species throughout their lifetime. Trees as oak, birch or willow for example, can host up to 200-300 insect herbivore species (Southwood 1961).

To find food sources and gain information about its quantity and quality, herbivorous animals use plant cues. Such cues can be visual (color, pattern and shape), olfactory or tactile (Schaefer and Ruxton 2011). Most plants emit a big variety of cues; which of these are used by animals to find host plants can depend on different factors as the animal's perception, its innate and learned preferences or the detectability of the cue (Schiestl and Johnson 2013; McLinn and Stephens 2006). Also, an animal may use different cues at different distance of approach or in different ecological contexts (Raguso 2008; Blarer et al. 2002).

Further, depending on the plant resource that animals consume and the consequential fitness outcome for plants, plant-animal interactions can be classified as mutualism (+/+), like for example pollination or seed dispersal, or antagonism (+/-) like folivory and florivory (Price 2002). Because of their effect on plant fitness, both mutualists and antagonists can impose selection on plant traits. Such selection imposed by plant-animal interactions can lead to the evolution of plant traits, differentiation within plant species and even species diversification (e.g. Gervasi and Schiestl 2017; Galen and Newport 1987; Peakall et al. 2010; Rieseberg and Willis 2007).

POLLINATION

Sessility in plants creates an obstacle for mate search. In the majority of plants this obstacle has been alleviated by mutualistic interactions with animals that transport pollen between plants (Eriksson and Bremer 1992; Ollerton et al. 2011). In fact the most important pollinators belong to the three extant animal groups that evolved flight: insects, birds and bats. Different pollinator species can range from specialists that feed only on little, often related plant species to generalists that feed on many species. For example, honeybees visit many different plant species to search for nectar and pollen (Westerkamp 1991) while each fig species is pollinated by a different species of fig wasp in a highly specialized interaction (Wiebes 1979). Plants profit from pollination by reproduction with conspecifics while pollinators mostly benefit by receiving floral rewards which can be in the form of food, shelter, mating sites, material for nest production or olfactory sexual attractants (Simpson and Neff 1981). Such reciprocal exploitation holds an underlying evolutionary conflict: The optimal situation for plants is to receive pollination service but save the costs of reward production, while for pollinators it is optimal to maximize the efficiency of reward exploitation irrespective of pollination service. Selection for the maximization of benefits and minimization of costs resulted in the occurrence of deceptive plant species that do not reward pollinators but exploit their preferences for floral cues to nevertheless receive pollination (Jersáková et al. 2006; Vereecken et al. 2012). On the other side, most plant species are visited by various pollinators (the pollinator guild) (Waser et al. 1996), of which many flower-visitors are poor pollinators, but nevertheless consume floral rewards (e.g. Rader et al. 2009). The composition of the pollinator guild, however, can vary temporally and spatially; A given pairwise plant-pollinator interaction can therefore be mutualistic in one ecological context but

antagonistic in another, depending on the presence of more efficient pollinators (Thompson and Fernandez 2006; Thompson and Cunningham 2002).

Plants are under selection to maximize intraspecific pollen transfer which promotes the attraction of specific and efficient pollinators. Pollinator attraction can be influences by floral cues as display, floral size, color and scent. Which cues attract or deter specific floral visitors can depend on the pollinator's perception and its innate and learned preferences (Schiestl and Johnson 2013). Adaptive innate preferences are the outcome of selection for efficient food search and can play an important role in specialized pollinators (Milet-Pinheiro et al. 2012). In contrast, in generalist pollinators associative learning can be more relevant (Gumbert 2000) as it allows for the alteration of innate preferences and the temporary adjustment of pollinator behavior to the most rewarding species in the present plant community. Thus, the information content a floral cue provides about floral rewards can be essential for the development of learned preferences (Knauer and Schiestl 2015; Blarer et al. 2002). However, plants can also exploit pollinator preferences that have evolved in a different ecological context than flower visitation (receiver bias) (Schiestl and Johnson 2013). For example, many Ophrys species emit species-specific insect sexual pheromones to attract males which pollinate the flowers during pseudocopulation (Schiestl et al. 1999).

Further, selection for an efficient intraspecific pollen transfer can also act on floral traits not involved in pollinator attraction. The efficiency of pollen transfer depends on the mechanical fit with pollinators and thus floral morphology (Cresswell 1998). For example, bilateral floral symmetry (zygomorphy) can affect pollinator orientation and increase floral constancy in generalist pollinators which supports the efficiency and specificity of pollination (Neal et al. 1998). Additionally, zygomorphy may select a narrower spectrum of more effective pollinators by reducing mechanical

fit with other visitors (Sargent 2004). Selection should further act against self-fertilization which favours inbreeding depression and pollen discounting. Physiological self-incompatibility and temporal (dichogamy) and spatial (herkogamy) separation of female and male functions of flowers are floral adaptations that mediate outcrossing in hermaphroditic plants (Barrett 1998, 2003).

These manifold selective pressures imposed by pollinators can drive the evolution of heritable floral traits and result in their convergence in unrelated species (pollinator syndromes). Pollinators can be divided into functional groups based on the similarity of selection pressure they exert on flowers. This resulted in the evolution of similar floral traits in plant species that specialized to the same functional group (Fenster et al. 2004; Rosas-Guerrero et al. 2014). For example, hummingbird-pollinated flowers are often characterized by reddish, scentless and tubular flowers with diurnal aperture, while bat-pollinated species typically have light colored flowers with a strong scent and nocturnal aperture (Rosas-Guerrero et al. 2014). Further, adaptation to different pollinators can contribute to reproductive isolation as pollinator shift directly result in reproductive barriers between populations. Pollinator-mediated selection has thus been related to sympatric and allopatric ecological speciation (Givnish 2010; Whittall and Hodges 2007) and, as a consequence, angiosperm diversification is higher in animal-pollinated than in wind-pollinated taxa (Kay and Sargent 2009). Also, speciation rates increase with the degree of pollinator specialization (Schiestl and Schlueter 2009) and can be favoured by the presence of specific floral traits that favour pollinator shifts. Plant lineages with nectar spurs for example, show higher species diversification compared to lineages without spurs (Hodges 1997).

HERBIVORY

Herbivory can negatively affect plant fitness in two ways: First, consumption of flowers, seeds or seedlings reduces fitness directly. Second, consumption of other plant tissues reduces fitness indirectly through reduced resource allocation by damaged tissues and trade-offs between reproduction and defense (McCall and Irwin 2006; Schiestl et al. 2014; Mole 1994). Herbivores have evolved various feeding strategies to obtain nutrients from plants. For example, leaf-eating beetles and caterpillars have mouthparts for chewing or snipping, thrips and spider mites have piercing-sucking tube-like structures while leafminers consume soft tissue between epidermal cells. Accordingly, herbivores often feed only on certain plant parts like roots, stem, leaves or flowers. Depending on the consumed part the fitness outcome for plants can differ drastically. Granivory, for example, causes complete loss of fitness, while folivory still allows for offspring production (Strauss and Zangerl 2002; Schoonhoven et al. 2005).

While herbivorous mammals usually feed on a large number of plant species, insect herbivores show the whole range from generalists to specialists that only feed on one host plant (Bernays and Graham 1988). The Oregon silverspot butterfly (*Speyeria zerene hippolyta*), for example, only feeds on *Viola adunca.* Oligophagous insects in contrast, feed on a number of plant species that all belong to the same plant family. The cabbage butterfly (*Pieris brassicae*), for example, is restricted to plant species from the Brassicaceae family. Further, polyphagous insects accept many different plant species from different families. The green peach aphid (*Myzus persicae*), for instance, has been recorded on more than 50 host plant species (Schoonhoven et al. 2005). While generalists profit from a bigger offer and smaller fluctuations in food availability, specialists are better in finding host plants because they can adapt to taxon-specific cues. Generalists in contrast, must have bigger

neural abilities to be able to respond to the various cues emitted by their host plants (Bernays 2001). Also, specialists are often better in extracting resources from their host plants (Scriber and Feeny 1979) and are more tolerant to the plants resistance. In some cases, specialists can even sequester plant resistance molecules and profit from enemy-free spaces. Caterpillars of *Utetheisa ornatrix,* for instance, are monophagous on *Crotalaria* from which they obtain pyrrolizidine alkaloids for defense (Bernays et al. 2003). Potentially as a result of these advantages and constraints, some degree of specialization evolved in the majority of insect herbivores (Bernays and Graham 1988).

To protect themselves from the negative effects of herbivory, plants evolved various adaptations (plant defense). Plant defense can be divided into resistance, which are traits that reduce herbivore preference or performance (Howe and Jander 2008; Hanley et al. 2007), and tolerance, which reduces the degree to which plant fitness is affected by herbivory (Strauss and Agrawal 1999). Herbivore resistance involves structural (e.g. spines and thornes), chemical (e.g. toxins) and phonological traits (e.g. rapid turnover of vulnerable parts). Some defensive traits are expressed constitutively irrespective of herbivore damage (constitutive defense), but occasionally plants can recognize herbivore attack (e.g. by compounds in insect oral secretions) and respond to it by inducing defensive traits (induced defense) (Karban 2011; Howe and Jander 2008). In *Cucumis sativus* for example, the cucurbitacin content in leaves increases after spider mite feeding (Agrawal et al. 1999). Herbivore-tolerant plants, in contrast, react to feeding damage by compensating the plant tissue to some degree. Increased branching, for example, or decreased leaf longevity are traits that can mediate compensation (Strauss and Agrawal 1999). Further, plants can defend themselves indirectly by involving the natural enemies of the herbivores. In some cases, plants constitutively reward predators with food

bodies or dormatia and in return profit by increased predator densities and herbivore repellence. Such association have been documented mainly with ants but also predatory mites (Heil 2008). Further, indirect defense can also be induced after insect feeding, either by the release of volatile organic compounds or the secretion of extrafloral nectar. Induced volatiles attract predators or parasitoids that rely on the herbivores as food source and profit from the induced cues by short searching times as herbivores themselves are often protected by camouflage (Dicke 2009). For example, spider mite infestation in lima beans induces five different volatile compounds out of which four attract predatory mites (Takabayashi et al. 1994). Induced extrafloral nectar in contrast, mostly attracts ants that defend this food source against putative competitors including herbivores. But also predatory mites, spiders or ladybeetles can consume extrafloral nectar and defend plants in return (Heil 2008).

CONTEXT DEPENDENCE OF PLANT-ANIMAL INTERACTIONS

Throughout their lifetime most plant species interact with various mutualists and antagonists that shape the evolution of plant traits (e.g. Schemske and Bradshaw 1999; Gómez et al. 2015; Cornell and Hawkins 2003; Rosas-Guerrero et al. 2014). Both, floral traits that increase the attractiveness of flowers to pollinators or pollination efficiency as well as traits improving plant defense or tolerance against herbivores are normally under positive directional selection (Medel et al. 2003; Gómez 2003; McCall et al. 2013; Strauss and Whittall 2006; Galen 1989; Sahli and Conner 2011). The role of plant traits in certain plant-animal interactions and the resulting selection on these traits, however, can depend on the context in which the interaction takes place (Strauss and Irwin 2004).

The preferences for floral cues in pollinators and herbivores are often adjusted to variable environments by learning. First, an animal's preference for a certain trait can depend on the trait's association with the desired resource. Bumble bees for example, develop preferences for floral cues that are associated with floral nectar (Blarer et al. 2002). Further, different animal species that interact with the same plant species may also interact with each other directly. Such direct interactions could cause a change in behavior and affect the selection imposed on plant traits. For example, some pollinators can deter others from flowers (Roubik 1978; Thomson 2004) which may cause changes in preferences for floral traits in the deterred species. Also, the composition of co-occurring plant species can affect animal preferences for certain traits. For example, after bumble bees developed preferences for specific colors that are associated with reward, they choose novel colors according to their similarity with the learned one (Gumbert 2000). Plant species therefore profit from co-flowering species with high rewards and similar floral cues which led to the evolution of Batesian floral mimicry in food-deceptive orchids (Jersáková et al. 2006).

Further, the selection imposed by one plant-animal interaction can depend on the presence of other interactions (Strauss and Irwin 2004). Many floral signals like color and scent have evolved to attract mutualists, mostly pollinators (Schiestl and Johnson 2013). However, antagonists can eavesdrop on such signals and use them to their own advantage. Such eavesdropping can cause a trade off for the plant between the attraction of mutualists and the avoidance of antagonists (Theis and Adler 2012; Schiestl et al. 2011; Brody and Mitchell 1997) resulting in conflicting selection on the same trait (Figure 1) (Gomez 2003; Gómez 2008). Similarly, plants can experience trade-offs between direct and indirect defense, namely when chemical defense molecules are not only toxic for herbivores but also for their natural

enemies. In cucumber plants (*Cucumis sativus*), the toxin cucurbitacin reduces herbivore survival but also lowers fecundity of predatory mites, which, accordingly, prefer infested plants with low cucurbitacin concentrations (Agrawal et al. 2002; Balkema-Boomstra et al. 2003).

However, even when pollinators and herbivores mediate selection on different traits, these selective pressures can still influence each other. When traits are correlated, selection pressures on these traits are not independent (Strauss and Irwin 2004). Also, some combinations of traits can be favoured at the expense of other possible combinations (correlational selection). In plant-animal interactions, combinations of floral signals causing high mutualist but low antagonist attraction at the same time should be under correlational selection (Herrera et al. 2002; Gómez 2008). Finally, the presence of one species can affect the selection imposed by another species on plant traits resulting in nonadditive selection (Figure 1) (Strauss and Irwin 2004). The degree of pollen limitation for example, can influence the effect of herbivores on plant fitness (Herrera et al. 2002; Gomez 2005) and consequentially their potential to impose selection on plant traits.

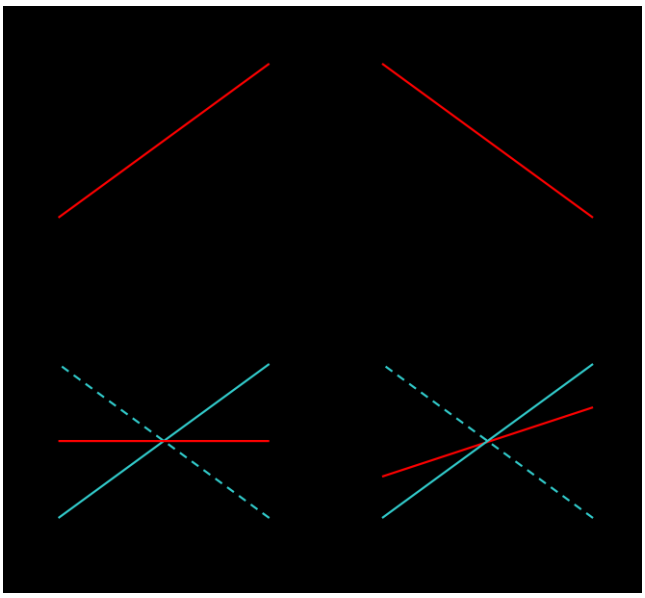


Figure 1 Different types of selection on floral traits imposed by plant-animal interactions. Red lines represent net selection, blue lines represent selection imposed by specific interactions. (A) positive directional selection, (B) negative directional selection, (C) conflicting additive selection by different interactions, (D) conflicting nonadditive selection by different interactions.

AIM OF THIS THESIS

So far most studies focused on the investigation of single pairwise plant-animal interactions and the individual role of specific floral signals in these interactions. In contrast, the interplay between different plant-animal interactions and the cumulative effects of different signals in the communication between plants and animals gained less attention. Thus, the purpose of this thesis was the investigation of the context-dependence of plant-animal interactions and the respective consequences for floral trait evolution.

To address our questions we used two species of the Brassicaceae family as a study system; *Brassica rapa* and *Biscutella laevigata*. Both species are characterized by a generalized pollination system with generalist bees as the main pollinators. Further, there are several folivorous and florivorous Lepidopteran species that have specialized on the Brassicaceae and are able to cope with their chemical defense.

In *chapter I and II* we focused on the effect of trait combinations on pollinator-mediated selection and floral signal evolution. Specifically we focused on so called honest floral signals – signals that are correlated to floral rewards. We investigated the relevance of signal honesty on the development of preferences in generalist pollinators and how pollinator behavior and floral constraints contribute to the maintenance of signal honesty. Finally, we developed a conclusive model on the evolution of honest floral signals.

In *chapter III and IV* in contrast, we focused on the interdependencies between different plant-animal interactions. First, we studied the effect of animal interactors on plant fitness in different environments. Specifically, we tested the net fitness effect of a generalist predator depending on the presence of herbivores as well as the effect of a pollinating herbivore depending on the presence of a more efficient pollinator.

Second, we investigated how the presence of one plant-animal interaction affects the selection mediated by another interaction by testing for correlational, conflicting and nonaddtive selection on floral traits by mutualists and antagonists.

REFERENCES

Agrawal AA, Gorski PM, Tallamy DW (1999) Polymorphism in plant defense against herbivory: Constitutive and induced resistance in Cucumis sativus. Journal of Chemical Ecology 25 (10):2285-2304. doi:10.1023/a:1020821823794

Agrawal AA, Janssen A, Bruin J, Posthumus MA, Sabelis MW (2002) An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecology Letters 5 (3):377-385. doi:10.1046/j.1461-0248.2002.00325.x

Balkema-Boomstra AG, Zijlstra S, Verstappen FWA, Inggamer H, Mercke PE, Jongsma MA, Bouwmeester HJ (2003) Role of cucurbitacin C in resistance to spider mite (Tetranychus urticae) in cucumber (Cucumis sativus L.). Journal of Chemical Ecology 29 (1):225-235. doi:10.1023/a:1021945101308

Barrett SCH (1998) The evolution of mating strategies in flowering plants. Trends Plant Sci 3 (9):335-341. doi:10.1016/s1360-1385(98)01299-0

Barrett SCH (2003) Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 358 (1434):991-1004. doi:10.1098/rstb.2003.1301

Bernays E, Graham M (1988) On the evolution of host specificity in phytophagous arthropods Ecology 69 (4):886-892. doi:10.2307/1941237

Bernays EA (2001) Neural limitations in phytophagous insects: Implications for diet breadth and evolution of host affiliation. Annual Review of Entomology 46:703-727. doi:10.1146/annurev.ento.46.1.703

Bernays EA, Chapman RF, Lamunyon CW, Hartmann T (2003) Taste receptors for pyrrolizidine alkaloids in a monophagous caterpillar. Journal of Chemical Ecology 29 (7):1709-1722. doi:10.1023/a:1024239201198

Blarer A, Keasar T, Shmida A (2002) Possible mechanisms for the formation of flower size preferences by foraging bumblebees. Ethology 108 (4):341-351. doi:10.1046/j.1439-0310.2002.00778.x

Brodie ED (1999) Predator-prey arms races. Bioscience 49 (7):557-568. doi:10.2307/1313476

Brody AK, Mitchell RJ (1997) Effects of experimental manipulation of inflorescence size on pollination and pre-dispersal seed predation in the hummingbird-pollinated plant Ipomopsis aggregata. Oecologia 110 (1):86-93. doi:10.1007/s004420050136

Clusella Trullas S, van Wyk JH, Spotila JR (2007) Thermal melanism in ectotherms. Journal of Thermal Biology 32 (5):235-245. doi:10.1016/j.jtherbio.2007.01.003

Cornell HV, Hawkins BA (2003) Herbivore responses to plant secondary compounds: A test of phytochemical coevolution theory. American Naturalist 161 (4):507-522. doi:10.1086/368346

Cresswell JE (1998) Stabilizing selection and the structural variability of flowers within species. Annals of Botany 81 (4):463-473. doi:10.1006/anbo.1998.0594

Darwin C (1859) On the origin of species by means of natural selection. London: John Murray

Dicke M (2009) Behavioural and community ecology of plants that cry for help. Plant Cell and Environment 32 (6):654-665. doi:10.1111/j.1365-3040.2008.01913.x

Eriksson O, Bremer B (1992) Pollination systems, dispersal modes, life forms, and diversification rates in angiosperm families Evolution 46 (1):258-266. doi:10.2307/2409820

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD (2004) Pollination syndromes and floral specialization. Annual Review of Ecology Evolution and Systematics 35:375-403. doi:10.1146/annurev.ecolsys.34.011802.132347

Galen C (1989) Measuring pollinator-mediated selection on morphometric floral traits - Bumble bees and the alpine sky pilot, Polemonium viscosum Evolution 43 (4):882-890. doi:10.2307/2409315

Galen C, Newport MEA (1987) Bumble bee behavior and selection on flower size in the sky pilot, Polemonium viscosum Oecologia 74 (1):20-23. doi:10.1007/bf00377340

Gervasi DDL, Schiestl FP (2017) Real-time divergent evolution in plants driven by pollinators. Nature Communications 8. doi:1469110.1038/ncomms14691
Givnish TJ (2010) Ecology of plant speciation. Taxon 59 (5):1326-1366
Gomez JM (2003) Herbivory reduces the strength of pollinator-mediated selection in the Mediterranean herb Erysimum mediohispanicum: Consequences for plant specialization. American Naturalist 162 (2):242-256
Gomez JM (2005) Non-additive effects of herbivores and pollinators on Erysimum mediohispanicum (Cruciferae) fitness. Oecologia 143 (3):412-418. doi:10.1007/s00442-004-1809-7
Gómez JM (2003) Herbivory reduces the strength of pollinator-mediated selection in the Mediterranean herb Erysimum mediohispanicum: Consequences for plant specialization. American Naturalist 162 (2):242-256. doi:10.1086/376574
Gómez JM (2008) Sequential conflicting selection due to multispecific interactions triggers evolutionary trade-offs in a monocarpic herb. Evolution 62 (3):668-679. doi:10.1111/j.1558-5646.2007.00312.x
Gómez JM, Perfectti F, Lorite J (2015) The role of pollinators in floral diversification in a clade of generalist flowers. Evolution 69 (4):863-878. doi:10.1111/evo.12632
Grant PR (1999) Ecology and evolution of Darwin's finches. Princeton, Princeton university press
Gumbert A (2000) Color choices by bumble bees (Bombus terrestris): innate preferences and generalization after learning. Behavioral Ecology and Sociobiology 48 (1):36-43. doi:10.1007/s002650000213
Hanley ME, Lamont BB, Fairbanks MM, Rafferty CM (2007) Plant structural traits and their role in anti-herbivore defence. Perspectives in Plant Ecology Evolution and Systematics 8 (4):157-178. doi:10.1016/j.ppees.2007.01.001
Heil M (2008) Indirect defence via tritrophic interactions. New Phytologist 178 (1):41-61. doi:10.1111/j.1469-8137.2007.02330.x
Herrera CM, Medrano M, Rey PJ, Sanchez-Lafuente AM, Garcia MB, Guitian J, Manzaneda AJ (2002) Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. Proceedings of the National Academy of Sciences of the United States of America 99 (26):16823-16828. doi:10.1073/pnas.252362799
Hodges SA (1997) Floral nectar spurs and diversification. International Journal of Plant Sciences 158 (6):S81-S88. doi:10.1086/297508
Howe GA, Jander G (2008) Plant immunity to insect herbivores. In: Annual Review of Plant Biology, vol 59. Annual Review of Plant Biology. pp 41-66. doi:10.1146/annurev.arplant.59.032607.092825
Jersáková J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. Biological Reviews 81 (2):219-235. doi:10.1017/s1464793105006986
Karban R (2011) The ecology and evolution of induced resistance against herbivores. Functional Ecology 25 (2):339-347. doi:10.1111/j.1365-2435.2010.01789.x
Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. Ecology Letters 7 (12):1225-1241. doi:10.1111/j.1461-0248.2004.00684.x
Kay KM, Sargent RD (2009) The Role of Animal Pollination in Plant Speciation: Integrating Ecology, Geography, and Genetics. In: Annual Review of Ecology Evolution and Systematics, vol 40. Annual Review of Ecology Evolution and Systematics. pp 637-656. doi:10.1146/annurev.ecolsys.110308.120310
Knauer AC, Schiestl FP (2015) Bees use honest floral signals as indicators of reward when visiting flowers. Ecology Letters 18 (2):135-143. doi:10.1111/ele.12386
Lande R, Arnold SJ (1983) The measurement of selection on correlated characters Evolution 37 (6):1210-1226. doi:10.2307/2408842
Leimu R, Fischer M (2008) A Meta-Analysis of Local Adaptation in Plants. Plos One 3 (12). doi:e4010 10.1371/journal.pone.0004010
McCall AC, Irwin RE (2006) Florivory: the intersection of pollination and herbivory. Ecology Letters 9 (12):1351-1365. doi:10.1111/j.1461-0248.2006.00975.x
McCall AC, Murphy SJ, Venner C, Brown M (2013) Florivores prefer white versus pink petal color morphs in wild radish, *Raphanus sativus*. Oecologia 172 (1):189-195. doi:10.1007/s00442-012-2480-z
McLinn CM, Stephens DW (2006) What makes information valuable: signal reliability and environmental uncertainty. Animal Behaviour 71:1119-1129. doi:10.1016/j.anbehav.2005.09.006
Medel R, Botto-Mahan C, Kalin-Arroyo M (2003) Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. Ecology 84 (7):1721-1732. doi:10.1890/01-0688

Milet-Pinheiro P, Ayasse M, Schlindwein C, Dobson HEM, Dotterl S (2012) Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. Behavioral Ecology 23 (3):531-538. doi:10.1093/beheco/arr219
Mole S (1994) Trade-offs and constraints in plant-herbivore defense theory - a life-history perspective Oikos 71 (1):3-12. doi:10.2307/3546166
Neal PR, Dafni A, Giurfa M (1998) Floral symmetry and its role in plant-pollinator systems: Terminology, distribution, and hypotheses. Annual Review of Ecology and Systematics 29:345-373. doi:10.1146/annurev.ecolsys.29.1.345
Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? Oikos 120 (3):321-326. doi:10.1111/j.1600-0706.2010.18644.x
Peakall R, Ebert D, Poldy J, Barrow RA, Francke W, Bower CC, Schiestl FP (2010) Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive Chiloglottis orchids: implications for pollinator-driven speciation. New Phytologist 188 (2):437-450. doi:10.1111/j.1469-8137.2010.03308.x
Price PW (2002) Species interactions and the evolution of biodiversity. Malden, Blackwell Science Ltd In: Plant-animal interactions:3-25
Rader R, Howlett BG, Cunningham SA, Westcott DA, Newstrom-Lloyd LE, Walker MK, Teulon DAJ, Edwards W (2009) Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. Journal of Applied Ecology 46 (5):1080-1087. doi:10.1111/j.1365-2664.2009.01700.x
Raguso RA (2008) Wake up and smell the roses: The ecology and evolution of floral scent. Annual Review of Ecology Evolution and Systematics 39:549-569. doi:10.1146/annurev.ecolsys.38.091206.095601
Rieseberg LH, Willis JH (2007) Plant speciation. Science 317 (5840):910-914. doi:10.1126/science.1137729
Rosas-Guerrero V, Aguilar R, Marten-Rodriguez S, Ashworth L, Lopezaraiza-Mikel M, Bastida JM, Quesada M (2014) A quantitative review of pollination syndromes: do floral traits predict effective pollinators? Ecology Letters 17 (3):388-400. doi:10.1111/ele.12224
Roubik DW (1978) Competitive interactions between neotropical pollinators and africanized honey bees Science 201 (4360):1030-1032. doi:10.1126/science.201.4360.1030
Sahli HF, Conner JK (2011) Testing for conflicting and nonadditive selection: Floral adaptation to multiple pollinators through male and female fitness. Evolution 65 (5):1457-1473. doi:10.1111/j.1558-5646.2011.01229.x
Sargent RD (2004) Floral symmetry affects speciation rates in angiosperms. Proceedings of the Royal Society B-Biological Sciences 271 (1539):603-608. doi:10.1098/rspb.2003.2644
Schaefer MH, Ruxton GD (2011) Plant-animal communication. New York, Oxford university press
Schemske DW, Bradshaw HD (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus). Proceedings of the National Academy of Sciences of the United States of America 96 (21):11910-11915. doi:10.1073/pnas.96.21.11910
Schiestl FP, Ayasse M, Paulus HF, Lofstedt C, Hansson BS, Ibarra F, Francke W (1999) Orchid pollination by sexual swindle. Nature 399 (6735):421-422. doi:10.1038/20829
Schiestl FP, Huber FK, Gómez JM (2011) Phenotypic selection on floral scent: trade-off between attraction and deterrence? Evolutionary Ecology 25 (2):237-248. doi:10.1007/s10682-010-9409-y
Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28 (5):307-315. doi:10.1016/j.tree.2013.01.019
Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. New Phytologist 203 (1):257-266
Schiestl FP, Schlueter PM (2009) Floral Isolation, Specialized Pollination, and Pollinator Behavior in Orchids. In: Annual Review of Entomology, vol 54. Annual Review of Entomology. pp 425-446. doi:10.1146/annurev.ento.54.110807.090603
Schoonhoven LM, Van Loon JJA, Dicke M (2005) Insect-plant biology. New York, Oxford university press:5-13
Scriber JM, Feeny P (1979) Growth of herbivorous caterpillars in relation to feeding specialization and to the growth form of their food plants Ecology 60 (4):829-850. doi:10.2307/1936618
Simpson BB, Neff JL (1981) Floral rewards - alternatives to pollen and nectar Annals of the Missouri Botanical Garden 68 (2):301-322. doi:10.2307/2398800
Southwood TRE (1961) The number of species of insect associated with various trees Journal of Animal Ecology 30 (1):1-8. doi:10.2307/2109
Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. Trends in Ecology & Evolution 14 (5):179-185. doi:10.1016/s0169-5347(98)01576-6

Strauss SY, Irwin RE (2004) Ecological and evolutionary consequences of multispecies plant-animal interactions. Annual Review of Ecology Evolution and Systematics 35:435-466. doi:10.1146/annurev.ecolsys.35.112202.130215
Strauss SY, Whittall JB (2006) Non-pollinator agents of selection on floral traits. Ecology and evolution of flowers:120-138
Strauss SY, Zangerl AR (2002) Plant-insect interactions in terrestrial ecosystems. Malden, Blackwell Science Ltd In: Plant-animal interactions:77-106
Takabayashi J, Dicke M, Posthumus MA (1994) Volatile herbivore-induced terpenoids in plant mite interactions - variation caused by biotic and abiotic factors Journal of Chemical Ecology 20 (6):1329-1354. doi:10.1007/bf02059811
Theis N, Adler LS (2012) Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. Ecology 93 (2):430-435
Thompson JN, Cunningham BM (2002) Geographic structure and dynamics of coevolutionary selection. Nature 417 (6890):735-738. doi:10.1038/nature00810
Thompson JN, Fernandez CC (2006) Temporal dynamics of antagonism and mutualism in a geographically variable plant-insect interaction. Ecology 87 (1):103-112. doi:10.1890/05-0123
Thomson D (2004) Competitive interactions between the invasive European honey bee and native bumble bees. Ecology 85 (2):458-470. doi:10.1890/02-0626
Vereecken NJ, Wilson CA, Hotling S, Schulz S, Banketov SA, Mardulyn P (2012) Pre-adaptations and the evolution of pollination by sexual deception: Cope's rule of specialization revisited. Proceedings of the Royal Society B-Biological Sciences 279 (1748):4786-4794. doi:10.1098/rspb.2012.1804
Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in pollination systems, and why it matters. Ecology 77 (4):1043-1060
Westerkamp C (1991) Honeybees are poor pollinators - why? . Plant Systematics and Evolution 177 (1-2):71-75. doi:10.1007/bf00937827
Whittall JB, Hodges SA (2007) Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447 (7145):706-U712. doi:10.1038/nature05857
Wiebes JT (1979) Co-evolution of figs and their insect pollinators Annual Review of Ecology and Systematics 10:1-12. doi:10.1146/annurev.es.10.110179.000245
Zu P, Blanckenhorn WU, Schiestl FP (2016) Heritability of floral volatiles and pleiotropic responses to artificial selection in *Brassica rapa*. The New phytologist 209 (3):1208-1219. doi:10.1111/nph.13652

CHAPTER I

Bees use honest floral signals as indicators of reward when visiting flowers

Knauer, A.C. and Schiestl, F.P.

Department of Systematic and Evolutionary Botany, Zollikerstrasse 107, University of Zurich, 8008 Zurich, Switzerland

Contribution statement

The following measurements were done during the M.Sc.: the association between floral traits and reward, the behavioral assays with real flowers, the behavioral assays with artificial flowers testing for the attractiveness of phenylacetaldehyde, GC-EAD. The following measurements were done during Ph.D.: scent emission of different floral parts, behavioral assay testing for the attractiveness of honest signals.

ABSTRACT

Pollinators visit flowers for rewards and should therefore have a preference for floral signals that indicate reward status, so called ‘honest signals’. We investigated honest signalling in *Brassica rapa L.* and its relevance for the attraction of a generalized pollinator, the bumble bee *Bombus terrestris (L.)*. We found a positive association between reward amount (nectar sugar and pollen) and the floral scent compound phenylacetaldehyde. Bumble bees developed a preference for phenylacetaldehyde over other scent compounds after foraging on *B. rapa*. When foraging on artificial flowers scented with synthetic volatiles, bumble bees developed a preference for those specific compounds that honestly indicated reward status. These results show that the honesty of floral signals can play a key role in their attractiveness to pollinators. In plants, a genetic constraint, resource limitation in reward and signal production, and sanctions against cheaters may contribute to the evolution and maintenance of honest signalling.

INTRODUCTION

The honesty of signals and its role in communication has mainly been investigated in animals (Saunders 2009; Szamado 2011), but is also of high relevance in plant–pollinator interactions (Schaefer et al. 2004). Pollinators visit flowers for rewards and gain an advantage from specifically visiting plant individuals with high reward quality and quantity (Waddington & Holden 1979). To assess the potential gain from rewards, pollinators rely on floral signals, such as color, shape and scent, which may indicate the reward status of a plant. In plant-pollinator interactions, the validity of a floral signal should thus depends on the variability in reward amounts offered by different flowers as well as the association of the floral signal with the reward, and the accuracy by which pollinators can detect the signal (McLinn & Stephens 2006).

An honest floral signal should be correlated with reward quality or quantity. The most obvious of such honest floral signals are those that are emitted by the reward itself (Wright & Schiestl 2009). Both pollen and nectar can emit visual and/ or olfactory signals that indeed have been shown to play an important role in pollinator attraction (Dobson et al. 1999; Hansen et al. 2007; Howell & Alarcon 2007). Floral signals not directly emitted by rewards, on the other hand, can also be honest signals for pollinators, namely when they are positively correlated with reward quantity or quality. Such a relationship has been documented for corolla diameter and nectar or pollen amount (e.g. Stanton & Preston 1988). Such ‘indirect’ honest signalling could be maintained by a constraint (Juenger et al. 2000; Conner 2002) as well as pollinator-mediated selection (Stanton & Young 1994). In *Turnera ulmifolia* positive selection on signal accuracy, namely the correlation of flower size and nectar amount, has been found (Benitez-Vieyra et al. 2010) and was suggested as the mechanism causing adaptive divergence of honest signals among *Salvia* species with different pollinators (Benitez-Vieyra et al. 2014).

Much progress has been achieved recently in the identification of specific attractive floral signals. However, it is not always known why pollinators respond to these signals. In specialized pollination systems, sensory exploitation can be important in some systems (Vereecken & Schiestl 2008; Castillo et al. 2012), whereas in others, signals form a specific identification token of the food plant (Burger et al. 2010; Milet-Pinheiro et al. 2013). On the contrary, in generalized pollination systems, floral signals act as learning cues, enabling pollinators to find and identify a rewarding flower (Chittka & Raine 2006; Leonard et al. 2011). Generalist pollinators thus may develop a preference for 'honest' floral signals correlated to rewards, as short-term preferences acquired through associative learning are known to play a key role in flower visitation by generalized pollinators (Chittka et al. 1999; Smith et al. 2006). However, because many learning experiments have been conducted with artificial flowers, it is often unknown which signals pollinators learn in a natural flower-visitation context, and if they preferentially use signals that are associated to rewards. The use of such honest signals would make sense, but requires the ability to distinguish between honest and dishonest signals through learning. So far, many experiments have shown that bees are capable of distinguishing between rewarding and rewardless flowers when emitting different scents or colors (e.g. Blarer et al. 2002; Lynn et al. 2005; Kulahci et al. 2008). However, a pollinators' ability to distinguish between an honest signal correlated with reward and a dishonest signal uncorrelated with reward has not been experimentally investigated.

In this study, we investigated mechanisms of pollinator attraction in *Brassica rapa* (Brassicaceae), a self-incompatible, annual herb with a generalized pollination system (Watanabe et al. 2000; Rader et al. 2009). As a model pollinator, we used the generalized bumble bee *Bombus terrestris* (Apidae). We addressed the following

questions: (1) Which floral signals are correlated with the reward amount produced by flowers? (2) Does the attractiveness of a signal depend on its association with reward status? We performed gas chromatographic analyses with electro-antennographic detection (GC-EAD) and behavioral assays to identify floral signals attracting bumble bees to *B. rapa* flowers. Furthermore, we quantified the emission of floral signals from different floral parts, and tested for their association with reward amount. The importance of honest signalling for the attraction of bumble bees was assessed in learning bioassays with artificial flowers, where different signals were manipulated to be either honest or dishonest.

METHODS

Study organism

Brassica rapa is a self-incompatible, annual or biennial herb native to Eurasia with a generalized pollination system (Watanabe et al. 2000; Rader et al. 2009). Bees such as *Apis mellifera* (Apidae), *Bombus terrestris* (Apidae) and syrphid flies such as *Eristalis tenax* (Syrphidae) have been documented as the most efficient pollinators in terms of pollen deposition per visit (Rader et al. 2009). *B. rapa* seeds were obtained from a natural population (Maarssen, the Netherland), from about 100 plants and grown in a greenhouse in the Botanical Garden of the University of Zurich under standardized light, soil and watering conditions (light period: 16 h, temperature: 22°C) in autumn 2013 and spring 2014. All plants were grown under nettings to prevent insect visitation until experimental use. For all experiments we used plants in full flower.

A generalist pollinator of *B. rapa*, the bumble bee, *Bombus terrestris* (Apidae), was used for behavioral experiments and GC-EAD. *B. terrestris* colonies were purchased from Andermatt Biocontrol (Andermatt, Switzerland) and the hives were

positioned in the centre of a flight cage (1 x 1 x 1 m). Bumble bees were fed on pollen (collected by honey bees, Biorex, Ebnat-Kappel, Switzerland) and Biogluc solution (BIOGLUC, Westerlo, Belgium).

Floral volatile collection, chemical analysis and electrophysiology

A detailed description of these methods is available in the Supporting Information methods. Two different methods were used to collect floral volatiles: (1) Tenax headspace collection with a dynamic push-pull system and Tenax TA as absorbent, and (2) Porapak headspace collection with a dynamic pull system and Porapak Q as absorbent. Porapak headspace collection was only used to collect samples for GC-EAD. Chemical analysis of samples and quantification of compounds was conducted as described in Schiestl et al. (2014). GC-EAD (Schiestl & Marion-Poll 2002) of headspace samples was performed with antennae of 11 bumble bee workers (from two colonies: five and six individuals per colony). Compounds that elicited a response in more than 30% of the bees were counted as EAD-active. The low threshold was chosen because 'no response' in GC-EAD recordings does not necessarily mean a lack of olfactory receptors responsive for a given volatile, because of often low signal to noise ratio in GC-EAD.

Association between floral signals and reward

In 20 flowering *B. rapa* plants, we measured eight floral traits, namely amounts of the four EAD-active scent compounds, floral color and corolla diameter as well as nectar sugar amount and number of pollen grains. All measurements were conducted between 2 and 5 pm. For measurements of flower diameter, petal color and rewards, only flowers which had opened on the same day were used. Scent was collected for 60 min from whole inflorescences. Total amounts of compounds were divided by the

number of open flowers during scent collection to calculate the amount emitted by an individual flower. Flower diameter and petal color were measured for ten flowers per individual. To assess corolla area, we calculated the square of flower diameter. Mean values were calculated to get an estimate for the signal emitted by one flower. In addition, mean corolla area was multiplied by the number of open flowers to obtain a value for total corolla area per inflorescence. Petal color was measured using a fibre optic spectrophotometer (AvaSpec-2048, Avantes, Apeldoorn, the Netherlands) and a xenon-pulsed light source (AvaLight-XE, Avantes, Apeldoorn, the Netherlands). We recorded the percentage reflectance of the visual spectrum of bumble bees between 250 and 650 nm every 0.573 nm (Peitsch et al. 1992).

For the quantification of the rewards produced by flowers, pollen and nectar of 10 flowers per individual were collected. Nectar was collected with 5 μL micropipettes (Blaubrand, Wertheim, Germany) and transmitted to filter paper stored in silica gel. The sector on the filter paper containing the nectar was cut from the rest of the filter paper and nectar was eluted in 1 mL high-purity Mili-Q water by shaking the dilution for 90 min with 400 rpm at 60°C on a laboratory shaker. Afterwards, 50 μL of the solution was dried at 60°C and derivatized with 100 μL of a mixture of anhydrous pyridine (Fisher Scientific, Geel, Belgium), hexamethylsilazane (Sigma-Aldrich, Buchs, Switzerland) and trimethylchlorosilane (Sigma-Aldrich, Buchs, Switzerland) (10:5:3) as described in Sweeley et al. (1963) and Baskin & Bliss (1969). Subsequently, samples were run by GC-MS as described in Medeiros & Simoneit (2007) (see also Supporting Information methods). We calculated total sugar amounts per flower and inflorescence as the sum of all different sugars (fructose, glucose, sucrose and sorbitol).

To measure individual pollen amounts, we collected the anthers in 1 mL Tween 80 solution (Costa & Yang 2009). To dissolve all pollen grains from the

anthers and disperse them, samples were shaken for 2 h at 1000 rpm (MS3 basic, IKA, Staufen, Germany). Subsequently, the suspension was filtered through a 30 μm CellTrics disposable filter (Partec GmbH, Münster, Germany). Pollen amounts were quantified using flow cytometry (Moon et al. 2011) using a Cell Lab QuantaTM SC-MPL flow cytometer (Beckman Coulter, Fullerton, Canada) with a mercury arc lamp. We used electronic volume measurements (EV set at 86 V) and a flow rate of 10 μL min^{-1}. Flow-CheckTM Fluorospheres (10 μm diameter, Beckman Coulter, Fullerton, Canada) were used as a size standard and only particles with a diameter above 16 μm were considered for the calculation of pollen grain number (Sarkissian & Harder 2001). Estimated nectar sugar and pollen amounts per flower were multiplied by the number of flowers to get an estimate of the total amount of reward produced by an inflorescence.

Scent emitted by different floral parts

Floral scent was collected in the greenhouse from the following floral parts individually: anthers, filaments, pistil, petals, sepals and the receptacle. We used the same flowers for the sampling of different floral parts and dissected the flowers stepwise. Only fresh flowers that had opened on the same day were used. In addition, we collected scent from nectar using the same collection protocol as for floral parts. Nectar was collected using micropipettes (5 μL, Braubrand, Wertheim, Germany) and blotted onto filter paper (1 x 1 cm, Whatman, Bottmingen; Switzerland). Scent collection was then immediately commenced from the filter paper. Scent from filter paper only was collected as a control. For each sample 100 flowers from 10 plants (10 flowers per individual) were used. Further we collected scent from 10 inflorescence leaves (one leaf per individual) to assess potential scent compounds emitted by the stem and leaves of inflorescences. Finally, this sampling

procedure was repeated three times to get three samples of each plant tissue (30 plants totally). Scent was collected in the afternoon for 30 min. In addition, we collected scent from whole, unmanipulated inflorescences from another 20 plants during 60 min. Total amounts of compounds were divided by the number of flowers used for scent collection to calculate amounts per individual flower.

Behavioral experiments with real and artificial flowers

To assess the importance of signal honesty for pollinator attraction, we conducted two behavioral assays with artificial flowers (Bioassay 1 and 2) whereas we used a behavioral assay with real flowers to assess the relevance of signals emitted by rewards (Bioassay 3). Artificial flowers were constructed from yellow paper discs which were placed over Eppendorf tubes and subsequently fixed to green wire stands (20 cm height). Scent was added using grey rubber septa (Supleco, Bellefonte, PA, USA), which were placed inside the Eppendorf tube. Before being used in bioassays, septa were soaked in a solution of synthetic scent compounds in solvent for 1 h and dried at room temperature for 4 h; scent compounds were subsequently emitted at a constant rate. The concentrations of all synthetic compounds in the solution were adjusted so that the emission rates were in the range of *B. rapa* plants. Detailed descriptions of the artificial flowers, the scent solutions and the emission rates of septa are available in the Supporting Information methods (Bioassay 1: Table S1; Bioassay 2: Table S2). All behavioral experiments were conducted in a flight cage in which one bumble bee hive was kept. Bumble bee landings were counted as measure of attractiveness. Sample sizes are given below and/or indicated in the figures.

Bioassay 1: Attractiveness of phenylacetaldehyde

Because we found a preference for olfactory over visual signals in bumblebees (see Fig. S1) and the scent compound phenylacetaldehyde was found to be an honest signal for reward amount, we conducted two dual-choice bioassays to investigate if bumble bees can potentially recognize phenylacetaldehyde as an honest signal in the scent bouquet of *B. rapa* and show a preference for this compound over others after learning. We tested a rewardless artificial flower emitting phenylacetaldehyde against one emitting the bouquet of the remaining EAD-active compounds. This dual-choice bioassay was first conducted with flower-naive bumble bees and afterwards with bumble bees previously exposed to a set of 20 *B. rapa* plants. In total, one hive was used. Exposure to plants took place during 4 h before the bioassay which allowed the bees to learn the signals emitted by *B. rapa* and associate them with reward. During the dual-choice bioassays artificial flowers were presented to pollinators with a distance of 12 cm. The positions of the two artificial flowers were exchanged after each landing by a pollinator and the paper discs were replaced by fresh ones after three landings by bumble bees. We caught and marked each landed individual to assure that an individual bumble bee was not counted twice. Thus, one individual bumble bee was only used once in each experiment but could have participated in both experiments (flower-naive and exposed).

Bioassay 2: Attractiveness of honest signals

Because bumble bees showed a preference for phenylacetaldehyde only after exposure to *B. rapa* plants, we carried out a further bioassay to test if bees developed this preference only in response to the honesty of the signal and if another compound, when honestly signalling reward status, would subsequently be preferred as well. This bioassay was carried out with two aromatic scent compounds,

phenylacetaldehyde and p-anisaldehyde. We first tested a rewardless artificial flower emitting phenylacetaldehyde against one emitting p-anisaldehyde using flower-naive bumble bees. Second, we gave the bumble bees the possibility to learn to distinguish between honest and dishonest olfactory signals. To do so, we set-up four flower types containing four different concentrations of reward (Biogluc solution), 0, 33, 67 and 100% (which were added to the artificial flowers using 0.5 mL Eppendorf tubes placed in the bigger tubes carrying the paper corolla). All four flower types were equipped with septa emitting the two compounds, one in rates correlated with the sugar concentration (honest signal) and one at constant rate (dishonest signal). We conducted three learning phases with phenylacetaldehyde as the honest signal and p-anisaldehyde as the dishonest one, and three learning phases where it was reversed. Each series of learning phases was carried out with a different hive to avoid order effects (two hives were used in total). The scent compound used as the dishonest signal had in all flowers an emission rate equal to the highest emission rate when it was used as honest signal (Table S2). During the learning phase, four arrays of four artificial flowers each were set-up in the bumble bee cage with a distance of 50 cm. The four flowers in the array contained the four different concentrations of Biogluc solution. The flowers were arranged in a random order with a distance of 12 cm. Landings of bees on flowers were counted. After 10 min, the flowers were removed from the cage, the paper discs replaced and the flowers refilled with reward solution and set back in a different order. One learning phase consisted of five 10 min time intervals and was carried out during 1 day. After each learning phase, we immediately repeated the dual-choice bioassay to test for a change in preference through learning. During the dual-choice bioassays, artificial flowers were presented to pollinators with a distance of 12 cm, the positions of the two artificial flowers were exchanged after each landing by a pollinator and the paper discs were replaced by

fresh ones after three landings by bumble bees. The amounts of the two scent compounds used in the dual-choice bioassays were equivalent to their highest amounts used in the learning phase (Table S2). We caught each landed individual to assure that an individual bumble bee was not counted twice. The same individual may have participated in the dual-choice bioassay conducted during different days.

Bioassay 3: Signals emitted by anthers and nectar

We conducted two dual-choice bioassays with *B. terrestris* to assess the relative importance of rewards compared to other floral parts for pollinator attraction. We tested (1) flowers without anthers against complete flowers, and (2) flowers without nectar against complete flowers. The two flowers presented simultaneously were abscised from the same plant individual and had opened on the same day. To present a flower to pollinators it was fixed in a pipette tip, which was then attached to a green wire stand (20 cm height). The two flowers were presented to free flying pollinators with a distance of 12 cm. Nectar was removed using micropipettes (5 µL; Blaubrand, Wertheim, Germany). Anthers were abscised with fine scissors. After each landing by a pollinator, flowers were replaced by fresh ones (experiment (1): 25 flower pairs from 15 plants tested, experiment (2): 26 flower pairs from 19 plants tested). Before the onset of the experiment, bees were allowed to visit flowering *B. rapa* plants. We recorded every landing and caught each landed individual to assure that an individual bumble bee was not counted twice. Caught individuals were marked according to the experiment. Thus, one individual bumble bee was only used once in each experiment, but could have participated in both experiments. In total, we used three hives for this bioassay.

Data analysis

To test for pollinator preferences in dual-choice bioassays, we used binominal tests. To test for a change in preference in the learning phase of bioassay 2, the proportion of visits to each flower type per array and time interval were calculated and analysed using a two-way ANOVA with the proportion of visits as response and the flower type and the time interval as explanatory variables. Further we calculated two parameters for the first and last time interval: (1) the proportion of visits to the lowest rewarding artificial flowers and (2) the proportion of visits to the highest rewarding artificial flowers. These parameters were analysed using a t-test.

To test for an association between floral traits and the amount of reward, we calculated a separate linear model for each floral signal with the amount of reward as response variable and a certain floral signal as explanatory variable. To reduce the large number of color variables (total 688 variables), a principal component analysis (PCA) was conducted by a singular value decomposition of the data matrix. For further analysis we included the first three principal components, which cumulatively explained 92% of variance. To obtain normal distribution of residuals response variables were log-transformed. All analyses were conducted using R 2.15.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Electrophysiology

Of the 20 compounds found in the headspace bouquet of *Brassica rapa* flowers, four compounds consistently elicited a response in *B. terrestris* olfactory neurons: three aromatic compounds (phenylacetaldehyde, acetophenone and p-anisaldehyde) and the sesquiterpene a-farnesene (Fig. S2, Table 1). In addition, the two fatty acid derivatives nonanal and decanal elicited a response, but were not considered for

further analysis because the amounts in control samples collected from empty cylinders did not differ from amounts collected from inflorescences. There were no obvious qualitative differences in the responses of individuals from different *B. terrestris* hives (Table S3).

Association between floral traits and reward

On a per flower basis, the amount of phenylacetaldehyde was significantly associated with nectar sugar and pollen amount whereas corolla area was significantly associated with pollen amount only (Table 2, Fig. S3). Interestingly, both phenylacetaldehyde and corolla area were associated with nectar volume (data not shown), which is obviously a less accurate measure of reward. On a per inflorescence basis, the amount of phenylacetaldehyde, the amount of a-farnesene and corolla area were significantly associated with nectar sugar and pollen amount (Table 2).

Scent emitted by different floral parts

Floral volatiles collected from different floral parts differed qualitatively and quantitatively (Table 1). Of the four EAD-active compounds, two aromatic compounds (phenylacetaldehyde and p-anisaldehyde) were found to be mainly emitted from the filaments and the petals, whereas a-farnesene was primarily found in the petal and the pistil samples. Furthermore, the anthers were the only floral source of the aromatic compound acetophenone. However, acetophenone was found in higher amounts in leaves than in any of the floral parts (Table 1). Furthermore, we found two EAD-active compounds in the nectar samples collected (phenylacetaldehyde and acetophenone), but the amounts were considerably lower

than the ones emitted by whole flowers (Table 1). Thus, neither nectar nor pollen was the main source of any of the floral scent compounds.

Behavioral experiments with artificial and real flowers

Bioassay 1 and 2

When phenylacetaldehyde was tested against the bouquet of all other EAD-active compounds emitted by *B. rapa*, only bumble bees previously exposed to *B. rapa* plants preferred phenylacetaldehyde over the bouquet, whereas naive bumble bees did not show any preference (Fig. 1, see also Fig. S4). In dual-choice bioassays testing phenylacetaldehyde against p-anisaldehyde, flower-naive bumble bees did not show any preference. But after exposure to artificial flowers emitting one compound as honest, and the other one as dishonest signal, they subsequently preferred the honest signal, no matter if it was phenylacetaldehyde or p-anisaldehyde (Fig. 2a). During the learning phase there was a significant interaction between flower type and time (two-way ANOVA, phenylacetaldehyde honest signal: F_{12} = 4.495, P < 0.001; p-anisaldehyde honest signal: F_{12} = 3.425, P < 0.001). Whereas the percentages of landings to the highest rewarding flowers increased between the first and last time interval (two sample t-test, phenylacetaldehyde honest signal: $t_{21.5}$ = -4.0604, P < 0.001; p-anisaldehyde honest signal: $t_{21.9}$ = -4.6998, P < 0.001), the ones to the lowest rewarding flowers decreased (two sample t-test, phenylacetaldehyde honest signal: $t_{11.9}$ = 4.334, P < 0.001; p-anisaldehyde honest signal: $t_{19.1}$ = 4.628, P < 0.001; Fig. 2b). Together, these results show that bumble bees only develop a preference for phenylacetaldehyde when it is an honest signal.

Table 1: Absolute amounts of scent compounds (mean ± s.e.m.) collected from *Brassica rapa* inflorescences (in pg l^{-1} $flower^{-1}$), different floral parts (in pg l^{-1} $flower^{-1}$), nectar (in pg l^{-1} $flower^{-1}$) and leaves (in pg l^{-1} $leaf^{-1}$). EAD-active compounds are marked in grey.

Compound	Inflorescence	Floral part						Nectar	Leaf
		Filament	Anther	Pistil	Petal	Sepal	Receptacle		
Aromatic compounds									
Benzaldehyde	343 ± 49	227 ± 85	23 ± 5	16 ± 3	113 ± 39	15 ± 2	–	11 ± 2	–
Phenylacetaldehyde	1618 ± 306	2159 ± 755	112 ± 18	–	818 ± 363	–	–	4 ± 1	–
Acetophenone	50 ± 15	–	3 ± 1	–	–	–	–	5 ± 2	23 ± 4
Phenylethyl alcohol	96 ± 22	37 ± 13	–	–	–	–	–	–	–
Methyl benzoate	54 ±8	38 ± 8	–	–	–	–	283 ± 95	–	17 ± 8
p-Anisaldehyde	101 ± 20	114 ± 45	–	–	61 ± 21	–	–	–	–
Benzyl acetate	3 ± 1	–	–	8 ± 4	–	3 ± 1	17 ± 8	–	–
Methyl salicylate	13 ± 2	7 ± 2	–	–	13 ± 4	–	–	–	–
Phenylethyl acetate	5 ± 1	20 ± 11	–	–	–	–	3 ± 2	–	–
Fatty acid derivatives									
(Z)-3-Hexen-1-ol	87 ± 16	372 ± 226	–	123 ± 14	17 ± 4	490 ± 258	194 ± 66	–	352 ± 120
6-Methyl, 5-hepten-2-one	17 ± 4	–	11 ± 6	10 ± 5	8 ± 2	7 ± 1	–	–	–
(Z)-3-Hexenyl acetate	484 ± 115	789 ± 147	–	458 ± 88	60 ± 27	1710 ± 349	643 ± 165	–	17793 ± 3196
Terpenoids									
Limonene	35 ± 7	4 ± 2	–	–	4 ± 1	–	–	–	27 ± 9
Linalool	20 ± 2	34 ± 13	–	–	10 ± 4	–	–	–	–
α-Farnesene	247 ± 29	36 ± 8	–	60 ± 6	138 ± 48	36 ± 11	–	–	–
Nitrogen containing compounds									
Benzyl nitrile	63 ± 36	128 ± 29	19 ± 7	4 ± 3	40 ± 15	–	–	–	–
Indole	32 ± 9	50 ± 4	22 ± 5	6 ± 2	39 ± 18	–	–	–	–
Formanilide	300 ± 9	249 ± 60	47 ± 15	–	125 ± 62	–	–	–	–
Methyl anthranilate	23 ± 1	8 ± 3	7 ± 2	–	4 ± 1	–	–	–	–
Sulphur containing compounds									
1-Butene-4-isothiocyanate	307 ± 147	2326 ± 1533	56 ± 27	254 ± 64	21 ± 1	106 ± 15	1273 ± 247	15 ± 6	5191 ± 1934

Table 2: Summary of the linear models with reward as response variable and floral signals as explanatory variables. A principal component analysis (PCA) was conducted to reduce the large number of color variables. PC1 - PC3 represent the first three principal components, which cumulatively explained 92% of variance. Results are given for models fitting average traits produced by one flower and fitting total traits produced by an inflorescence.

Floral signal	Per flower						Per inflorescence					
	log (Number of pollen grains)			log (Sugar amount)			log (Number of pollen grains)			log (Sugar amount)		
	β ± s.e.	t	P	β ± s.e.	t	P	β ± s.e.	t	P	β ± s.e.	t	P
Phenylacetaldehyde	$1.5e^{-4} \pm 6.2e^{-5}$	2.440	**0.025**	$2.5e^{-2} \pm 9.2e^{-3}$	2.697	**0.015**	$1.2e^{-5} \pm 3.3e^{-6}$	3.603	**0.002**	$1.4e^{-5} \pm 3.7e^{-6}$	3.754	**0.002**
Acetophenone	$5.3e^{-4} \pm 1.4e^{-3}$	0.368	0.717	$-1.5e^{-1} \pm 2.1e^{-1}$	- 0.724	0.479	$-1.7e^{-5} \pm 5.7e^{-4}$	- 0.030	0.977	$-1.4e^{-4} \pm 7.0e^{-2}$	- 0.200	0.844
p-Anisaldehyde	$-1.5e^{-4} \pm 1.1e^{-3}$	- 0.137	0.893	$-9.9e^{-2} \pm 1.5e^{-1}$	- 0.628	0.538	$6.1e^{-5} \pm 1.0e^{-4}$	0.608	0.551	$7.8e^{-5} \pm 1.2e^{-4}$	0.676	0.508
α-Farnesene	$1.1e^{-3} \pm 7.1e^{-4}$	1.598	0.127	$1.6e^{-1} \pm 1.2e^{-1}$	1.380	0.185	$1.1e^{-4} \pm 3.3e^{-5}$	3.413	**0.003**	$1.2e^{-4} \pm 3.9e^{-5}$	3.168	**0.006**
Corolla area	$7.5e^{-3} \pm 2.8e^{-3}$	2.730	**0.014**	$5.9e^{-3} \pm 3.0e^{-3}$	1.980	0.064	$2.8e^{-4} \pm 4.8e^{-5}$	5.872	**< 0.001**	$3.6e^{-4} \pm 4.7e^{-5}$	7.632	**< 0.001**
Color-PC1	$-4.8e^{-4} \pm 5.4e^{-3}$	- 0.088	0.931	$5.1e^{-1} \pm 9.4e^{-1}$	0.541	0.596	–	–	–	–	–	–
Color-PC2	$-1.4e^{-3} \pm 6.4e^{-3}$	- 0.213	0.834	$-7.0e^{-1} \pm 9.6e^{-1}$	0.729	0.476	–	–	–	–	–	–
Color-PC3	$-9.6e^{-3} \pm 1.3e^{-2}$	- 0.772	0.450	1.0 ± 2.0	- 0.532	0.601	–	–	–	–	–	–

Bioassay 3

Bumble bees did not show a preference for complete flowers over flowers with abscised anthers or removed nectar in the dual-choice bioassays using single flowers (Fig. 3). Thus, signals involved in pollinator attraction are emitted neither by nectar nor by pollen directly.

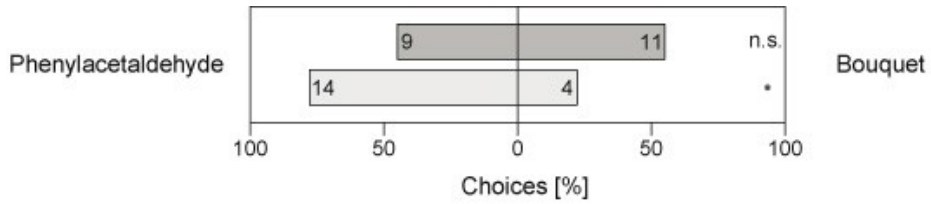


Figure 1 Dual-choice bioassays testing phenylacetaldehyde against the bouquet of the remaining EAD-active compounds. The graph shows percentages of landings by *Bombus terrestris* on artificial flowers. Dark grey: flower-naive; light grey: after exposure to 20 *B. rapa* plants. The numbers in the bars are the absolute numbers of landings. Flower-naive bumble bees didn't show a preference, but after exposure to *B. rapa* they preferred phenylacetaldehyde over the bouquet (Binomial test: n.s.: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).

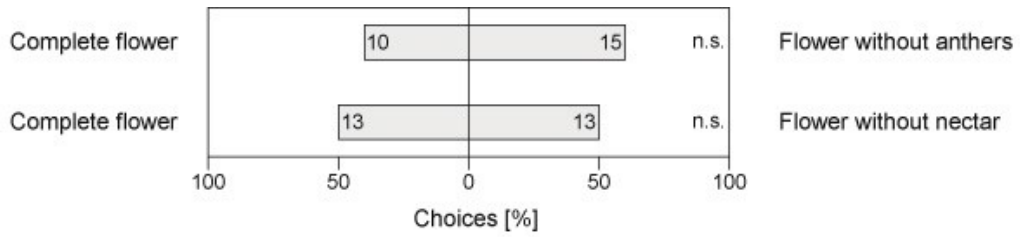


Figure 3: Dual-choice bioassays testing complete flowers against flowers without reward. The graph shows percentages of landings by *Bombus terrestris* on single flowers. The numbers in the bars are the absolute numbers of landings. Bumble bees didn't show a preference for complete flowers over flowers without reward (Binomial test: n.s.: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).

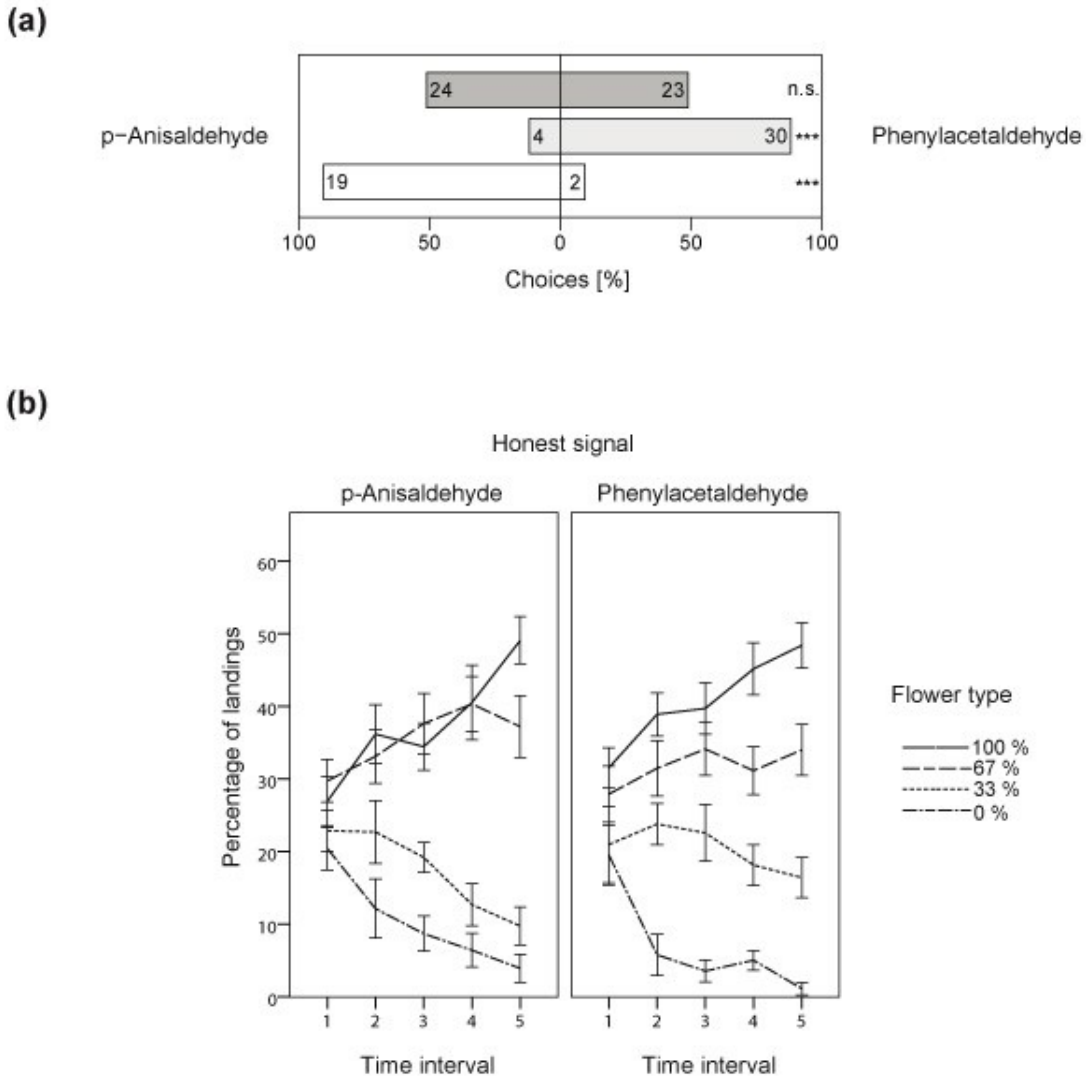


Figure 2: Percentages of landings by *Bombus terrestris* on artificial flowers. **(a)** Dual-choice bioassays testing phenylacetaldehyde against p-anisaldehyde. Dark grey: flower-naive; light grey: after learning phase with phenylacetaldehyde as honest signal and p-anisaldehyde as dishonest one; white: after learning phase with p-anisaldehyde as honest signal and phenylacetaldehyde as dishonest one. The numbers in the bars are the absolute numbers of landings. Flower-naive bumble bees didn't show a preference, but after exposure to artificial flowers they preferred the honest signal (Binomial test: n.s.: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). **(b)** Learning phase. The flower types (solid and hatched lines) refer to the concentration of Biogluc solution used as reward. Left side: Learning phase with p-anisaldehyde as honest signal and phenylacetaldehyde as dishonest one; right side: Learning phase with phenylacetaldehyde as honest signal and p-anisaldehyde as dishonest one.

DISCUSSION

Although honest signals are predicted to play a key role in plant-animal interactions, few studies have shown which signals can honestly indicate reward status of flowers and how they are used by pollinators. Our study demonstrates 'indirect' honest signalling through the scent compound phenylacetaldehyde for pollen and sugar amount on a per flower and a per inflorescence level. Furthermore, bumble bees develop a preference for phenylacetaldehyde after foraging on *B. rapa* individuals or artificial flowers that honestly signal with this scent compound. We also show with two synthetic scent compounds that bumble bees can distinguish between individual scent compounds that either signal honestly or dishonestly, and strongly prefer the honest signal independent of its chemical nature. Overall, our results provide novel insights into the use of honest signals by generalized pollinators, and how honest signalling evolves in plants.

Floral rewards are often concealed within a flower, and thus protected from direct visual inspection by pollinators. Signals directly emitted by rewards can, however, reliably indicate reward status (Raguso 2008) and some studies found them to play an important role in pollinator attraction (Dobson et al. 1999; Hansen et al. 2007; Howell & Alarcon 2007). In our study, we did not detect such 'direct' honest signals; although we found two EAD-active volatiles in nectar and anther samples, both compounds were emitted in higher amounts from other floral sources or leaves than from rewards. The two hydrophilic compounds in the nectar may thus be passively absorbed from surrounding tissues (Raguso 2004). The yellow color of pollen may also not be an effective honest signal, as it reflects in the same color spectrum as petals in *B. rapa* (Fig. S5; (Lunau et al. 2006)). The strongest evidence against direct honest signals comes from our bioassays showing that the removal of

neither anthers nor nectar had an effect on the initial attractiveness of *B. rapa* flowers to bumble bees.

Floral signals not exclusively emitted by rewards can still be associated with reward quantity or quality and thus represent honest signals for pollinators. The floral signal phenylacetaldehyde was positively correlated with the amount of reward (pollen and nectar sugar) in *B. rapa*. Whereas flower size has been shown in previous studies to be positively correlated with nectar and pollen amount (e.g. Stanton & Preston 1988), so far no study has reported a positive relationship between the amount of a scent compound and reward. This result shows that both, visual and olfactory signals can be 'indirect' honest signals to pollinators. The validity of a floral signal for a pollinator depends first of all on the variability in reward (McLinn & Stephens 2006). As we found considerable variation in pollen and sugar produced by the flowers of different plant individuals (Fig. S3), pollinators are likely to profit from distinguishing between flowers based on the amounts of reward they provide. Furthermore, the validity of the signals depends on the strengths of their correlations with reward (reliability) and the accuracy by which pollinators can assess the variation within a signal (detectability; McLinn & Stephens 2006). In general, variation in floral scent is often very high (Raguso 2008), and may typically exceed variation in morphological traits such as flower size. The phenylacetaldehyde emission in our study ranked between 1 and 273 ng per inflorescence. In *Polemonium viscosum*, bumble bees have been shown to distinguish between 2-phenylethanol emission rates of 10 ng L^{-1} and 35 ng L^{-1}per inflorescence (Galen et al. 2011). The differences in phenylacetaldehyde emission found in *B. rapa* plants should therefore be detectable by *B. terrestris*, possibly with higher precision than size differences, which may explain the preferential use of olfactory signals in foraging bumblebees (see Fig. S1).

Phenylacetaldehyde is an honest signal in *B. rapa*, and it is also preferentially used by bumble bees compared to other scent compounds, as they develop a preference for it over the rest of the floral scent bouquet after visiting flowering *B. rapa* plants. Our experiments show, however, that it is not the chemical nature of this compound that makes it so attractive, but its correlation with reward. When foraging on artificial flowers bumble bees quickly build-up a preference for either phenylacetaldehyde or p-anisaldehyde, when either one of them is correlated with reward. It seems thus likely that the bees can use any detectable signal correlated with reward to efficiently exploit flowers. Thus, bumble bees are not only capable of learning to associate a signal with a reward but also to evaluate the signals' information content concerning reward amount and to use this information to improve foraging efficiency.

In plants, little is known about the prerequisites for the evolution of honest signalling. We suggest three non-mutually exclusive mechanisms promoting honest floral signalling: genetic constraint', 'resource limitation in reward and signal production' and 'verification leading to sanction against cheaters' (Juenger et al. 2000; Hurd & Enquist 2005; Szamado 2011; Raihani et al. 2012; Broom et al. 2013). Under a genetic constraint, correlations between floral traits are generated by pleiotropy. Such a genetic constraint has been shown to cause the correlation among six floral traits in *Raphanus raphanistrum* (Conner 2002). In contrast, the resource limitation scenario predicts that honest signalling is maintained because the production of both, signal and reward, are costly and thus resource limited. Then, only individuals with a high resource allocation to flowers may be able to produce high signal values and large rewards. In *Epilobium angustifolium*, for example, flower size and nectar amount increase with watering (Carroll et al. 2001). Whereas nectar and pollen are usually costly for plants (Southwick 1984), little is known about the

costs of floral scent. Aromatic compounds like phenylacetaldehyde are, however, synthesized from phenylalanine (Dudareva et al. 2013) and may thus compete with the synthesis of other amino acid-based metabolites. Another possible mechanism maintaining honest signalling in this system is verification leading to sanctions against cheaters (Raihani et al. 2012). This scenario builds on the pollinator's ability to distinguish between honest and dishonest signals and to evaluate the reliability of honest signals. Sanctions occur when pollinators leave a cheating plant and avoid it subsequently. Thus, the impact of the sanctions is influenced by the need of the plant for repeated interactions with pollinators (Broom et al. 2013). Plants that typically depend on relatively high visitation rates by pollinators and the return of pollinators are annuals with high flower number, sequentially flowering inflorescences, inefficient pollination and self-incompatibility. Furthermore, generalist pollinators are more able to apply sanctions as they can visit a variety of different plant species but return more frequently to plants providing high amounts of reward (Makino & Sakai 2007). Judging from these criteria, *B. rapa* seems vulnerable to sanctions by pollinators, as it is annual, sequentially flowering, produces a large number of flowers, is selfincompatible and mainly pollinated by generalist pollinators (Watanabe et al. 2000; Rader et al. 2009). Thus sanctions by pollinators seem relevant in the maintenance of honest signalling. The opposite extreme in terms of pollination systems are orchids with their highly efficient pollination system through pollinia, often specific pollination and self-compatibility (Schiestl & Schlüter 2009). Indeed, orchids are the prime example for frequent evolution of cheating (Hobbhahn et al. 2013) supporting the idea of sanctions against cheaters being an important mechanism for the evolution of honest or dishonest signalling.

In conclusion, we show that honest signalling can be an important mechanism for the maintenance of mutualistic plant-pollinator associations. We suggest that

flowering and mating system as well as life history should be incorporated more into the study of floral signalling, to better understand patterns of evolution of honest and dishonest signalling mechanisms.

ACKNOWLEDGEMENTS

We would like to thank Edward Connor and Franz Huber for their support during scent collection and analysis. Markus Meierhofer and Rayko Jonas took good care of the here used plants. Robert Raguso provided helpful comments on an earlier version of the manuscript. Further we thank Christian Sailer for his introduction to the use of flow cytometry and Tom de Jong and Nicole van Dam for providing the *B. rapa* seeds. Peter Arnold and Gianna Petendi conducted a preliminary learning experiment with bumblebees. The research leading to these results has received funding from the European Union's Seventh Framework Program ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement n° 281093.

REFERENCES

Ashman TL, Bradburn M, Cole DH, Blaney BH, Raguso RA (2005) The scent of a male: The role of floral volatiles in pollination of a gender dimorphic plant. Ecology 86 (8):2099-2105. doi:10.1890/04-1161

Benitez-Vieyra S, Fornoni J, Pérez-Alquicira J, Boege K, Dominguez CA (2014) The evolution of signal – reward correlation in bee- and hummingbird-pollinated species of *Salvia*. Proceedings of the Royal Society B-Biological Sciences 281 (1782):20132934. doi:10.1098/rspb.2013.2934

Benitez-Vieyra S, Ordano M, Fornoni J, Boege K, Dominguez CA (2010) Selection on signal-reward correlation: limits and opportunities to the evolution of deceit in Turnera ulmifolia L. Journal of Evolutionary Biology 23 (12):2760-2767. doi:10.1111/j.1420-9101.2010.02132.x

Blarer A, Keasar T, Shmida A (2002) Possible mechanisms for the formation of flower size preferences by foraging bumblebees. Ethology 108 (4):341-351. doi:10.1046/j.1439-0310.2002.00778.x

Broom M, Ruxton GD, Schaefer HM (2013) Signal verification can promote reliable signalling. Proceedings of the Royal Society B-Biological Sciences 280 (1771). doi:2013156010.1098/rspb.2013.1560

Burger H, Dotterl S, Ayasse M (2010) Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Functional Ecology 24 (6):1234-1240. doi:10.1111/j.1365-2435.2010.01744.x

Chittka L, Raine NE (2006) Recognition of flowers by pollinators. Current Opinion in Plant Biology 9 (4):428-435

Chittka L, Thomson JD, Waser NM (1999) Flower constancy, insect psychology, and plant evolution. Naturwissenschaften 86 (8):361-377

Costa CM, Yang S (2009) Counting pollen grains using readily available, free image processing and analysis software. Annals of Botany 104 (5):1005-1010. doi:10.1093/aob/mcp186
Dobson HEM, Danielson EM, Van Wesep ID (1999) Pollen odor chemicals as modulators of bumble bee foraging on Rosa rugosa Thunb. (Rosaceae). Plant Species Biology 14 (2):153-166. doi:10.1046/j.1442-1984.1999.00020.x
Dudareva N, Klempien A, Muhlemann JK, Kaplan I (2013) Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytologist 198 (1):16-32. doi:10.1111/nph.12145
Frisch Kv (1919) Über den Geruchssinn der Bienen und seine blütenbiologische Bedeutung. Zool Jb Physiol 37:1-238
Galen C, Kaczorowski R, Todd SL, Geib J, Raguso RA (2011) Dosage-dependent impacts of a floral volatile compound on pollinators, larcenists, and the potential for floral evolution in the alpine skypilot Polemonium viscosum. American Naturalist 177 (2):258-272. doi:10.1086/657993
Gomez JM, Bosch J, Perfectti F, Fernandez JD, Abdelaziz M, Camacho JPM (2008) Association between floral traits and rewards in Erysimum mediohispanicum (Brassicaceae). Annals of Botany 101 (9):1413-1420. doi:10.1093/aob/mcn053
Hansen DM, Olesen JM, Mione T, Johnson SD, Mueller CB (2007) Coloured nectar: distribution, ecology, and evolution of an enigmatic floral trait. Biological Reviews 82 (1):83-111. doi:10.1111/j.1469-185X.2006.00005.x
Harder LD, Cruzan MB (1990) An evaluation of the physiological and evolutionary influences of inflorescence size and flower depth on nectar producion Functional Ecology 4 (4):559-572. doi:10.2307/2389323
Hobbhahn N, Johnson SD, Bytebier B, Yeung EC, Harder LD (2013) The evolution of floral nectaries in Disa (Orchidaceae: Disinae): recapitulation or diversifying innovation? Annals of Botany 112 (7):1303-1319. doi:10.1093/aob/mct197
Howell AD, Alarcon R (2007) Osmia bees (Hymenoptera : Megachilidae) can detect nectar-rewarding flowers using olfactory cues. Animal Behaviour 74:199-205. doi:10.1016/j.anbehav.2006.11.012
Hurd PL, Enquist M (2005) A strategic taxonomy of biological communication. Animal Behaviour 70:1155-1170. doi:10.1016/j.anbehav.2005.02.014
Kessler D, Baldwin IT (2007) Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of Nicotiana attenuata. Plant Journal 49 (5):840-854. doi:10.1111/j.1365-313X.2006.02995.x
Leonard AS, Dornhaus A, Papaj DR (2011) Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. Journal of Experimental Biology 214 (1):113-121. doi:10.1242/jeb.047407
Lunau K, Fieselmann G, Heuschen B, van de Loo A (2006) Visual targeting of components of floral colour patterns in flower-naive bumblebees (Bombus terrestris; Apidae). Naturwissenschaften 93 (7):325-328. doi:10.1007/s00114-006-0105-2
Makino TT, Sakai S (2007) Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. Functional Ecology 21 (5):854-863
Milet-Pinheiro P, Ayasse M, Dobson HEM, Schlindwein C, Francke W, Dotterl S (2013) The chemical basis of host-plant recognition in a specialized bee pollinator. J Chem Ecol 39 (11-12):1347-1360. doi:10.1007/s10886-013-0363-3
Moon HS, Eda S, Saxton AM, Ow DW, Stewart CN, Jr. (2011) An efficient and rapid transgenic pollen screening and detection method using flow cytometry. Biotechnology Journal 6 (1):118-123. doi:10.1002/biot.201000258
Pauw A, Stofberg J, Waterman RJ (2009) Flies and flowers in Darwin's race. Evolution 63 (1):268-279. doi:10.1111/j.1558-5646.2008.00547.x
Peitsch D, Fietz A, Hertel H, Desouza J, Ventura DF, Menzel R (1992) The spectral input system of hymenopteran insects and their receptor-based color-vision Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology 170 (1):23-40
Pyke GH (1978) Optimal foraging - movement patterns of bumblebees between inflorescences. Theoretical Population Biology 13 (1):72-98. doi:10.1016/0040-5809(78)90036-9
Rader R, Howlett BG, Cunningham SA, Westcott DA, Newstrom-Lloyd LE, Walker MK, Teulon DAJ, Edwards W (2009) Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. Journal of Applied Ecology 46 (5):1080-1087. doi:10.1111/j.1365-2664.2009.01700.x
Raguso RA (2004) Why are some floral nectars scented? Ecology 85 (6):1486-1494

Raguso RA (2008) Wake up and smell the roses: The ecology and evolution of floral scent. Annual Review of Ecology Evolution and Systematics 39:549-569. doi:10.1146/annurev.ecolsys.38.091206.095601

Raihani NJ, Thornton A, Bshary R (2012) Punishment and cooperation in nature. Trends in Ecology & Evolution 27 (5):288-295. doi:10.1016/j.tree.2011.12.004

Sarkissian TS, Harder LD (2001) Direct and indirect responses to selection on pollen size in Brassica rapa L. Journal of Evolutionary Biology 14 (3):456-468. doi:10.1046/j.1420-9101.2001.00285.x

Sasaki K, Takahashi T (2002) A flavonoid from Brassica rapa flower as the UV-absorbing nectar guide. Phytochemistry 61 (3):339-343. doi:Pii s0031-9422(02)00237-610.1016/s0031-9422(02)00237-6

Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28 (5):307-315. doi:10.1016/j.tree.2013.01.019

Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. New Phytologist 203 (1):257-266

Schiestl FP, Marion-Poll F (2002) Detection of physiologically active flower volatiles using gas chromatography coupled with electroantennography. Analysis of taste and aroma, vol Volume 21.

Schiestl FP, Schlüter PM (2009) Floral isolation, specialized pollination, and pollinator behavior in orchids. Annual Review of Entomology 54:425-446

Smith BH, Wright GA, Daly KC (2006) Learning-based recognition and discrimination of floral odors. In: Dudareva N, Pichersky E (eds) Biology of Floral Scent. Taylor&Francis, Boca Raton, pp 263-296

Southwick EE (1984) Photosynthate allocation to floral nectar - a neglected energy investment. Ecology 65 (6):1775-1779. doi:10.2307/1937773

Stanton ML, Preston RE (1988) Ecological consequences and phenotypic correlates of petal size variation in wild radish, Raphanus sativus (Brassicaceae) American Journal of Botany 75 (4):528-539. doi:10.2307/2444218

Szamado S (2011) The cost of honesty and the fallacy of the handicap principle. Animal Behaviour 81 (1):3-10. doi:10.1016/j.anbehav.2010.08.022

Vereecken NJ, Schiestl FP (2008) The evolution of imperfect floral mimicry. Proceedings of the National Academy of Sciences of the United States of America 105 (21):7484-7488. doi:10.1073/pnas.0800194105

von Arx M, Goyret J, Davidowitz G, Raguso RA (2012) Floral humidity as a reliable sensory cue for profitability assessment by nectar-foraging hawkmoths. Proceedings of the National Academy of Sciences of the United States of America 109 (24):9471-9476. doi:10.1073/pnas.1121624109

Waddington KD, Holden LR (1979) Optimal foraging - flower selection by bees. American Naturalist 114 (2):179-196. doi:10.1086/283467

Watanabe M, Ito A, Takada Y, Ninomiya C, Kakizaki T, Takahata Y, Hatakeyama K, Hinata K, Suzuki G, Takasaki T, Satta Y, Shiba H, Takayama S, Isogai A (2000) Highly divergent sequences of the pollen self-incompatibility (S) gene in class-I S haplotypes of Brassica campestris (syn. rapa) L. Febs Letters 473 (2):139-144. doi:10.1016/s0014-5793(00)01514-3

Wright GA, Schiestl FP (2009) The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. Functional Ecology 23 (5):841-851. doi:10.1111/j.1365-2435.2009.01627.x

Young HJ, Stanton ML (1990) Influences of floral variation on pollen removal and seed production in wild radish. Ecology 71 (2):536-547. doi:10.2307/1940307

SUPPORTING INFORMATION

METHODS

Floral volatile collection

Two different methods were used to collect floral volatiles: (1) Tenax headspace collection with a dynamic push-pull system and Tenax TA as absorbent, and (2) Porapak headspace collection with a dynamic pull system and Porapak Q as absorbent.

To collect scent from whole inflorescences, floral parts, nectar, leaves and rubber septa we used Tenax headspace collection. Plant material was enclosed in glass cylinders, featuring two openings (dimensions: 5 cm diameter, 25 cm height: scent collection for whole inflorescences and rubber septa; 3.5 cm diameter, 15 cm height: scent collection for floral parts, nectar and leaves; all glass cylinders were treated with sigmacoate (Sigma-Aldrich, Buchs, Switzerland)). The bottom of the cylinder was closed with a teflon plate, which consisted of two halfs fixed together with two screws and mounted on the margin of the glass cylinder. A central hole allowed for the insertion of a plant stem without injuring it. For volatile collection glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into one opening and attached to a Micro Air Sampler (PAS-500 Micro Air Sampler, Spectrex, Redwood City, CA, USA) with a silicon tube. Air was pulled through the glass tubes at a flow rate of 200 ml min^{-1}. A charcoal filter (small, matrix activated coconut charcoal, 20-40 mesh, Bed A 100 mg, Bed B 50 mg, Sigma-Aldrich, Mexico) was inserted through the other opening and air was pushed through this filter into the cylinder at a rate of 150 ml min^{-1} using a membrane pump (Air pump 400, Eheim, Deizisau, Germany). As a control for ambient contaminants we collected scent from an empty glass cylinder using the same collection parameters. Further we

collected scent from a glass cylinder with a filter paper only as a control for contaminations emitted by the filter paper which was used for scent collection from nectar. After scent collection the Tenax tubes were stored at -30˚C until gas chromatographic analysis.

To collect floral scent from whole inflorescences for gas chromatography-electroantennographic detection (GC-EAD) we used Porapak headspace collection (Huber et al. 2005). Inflorescences were enclosed in the same glass cylinders described above, and a glass tube filled with Porapak Q was placed inside each cylinder. The glass tubes were filled with 4 mg of Porapak Q (Mesh size 80/100; Alltech Associates Inc., Deerfield, IL, USA) enclosed by a layer of quartz wool and glass beads (0.3 mm, Merck KGaA, Darmstadt, Germany) fused into the glass tube by heating on both sides. Before use, all Porapak tubes were cleaned with 400 µl dichloromethane. The tubes were connected to a Micro Air Sampler (PAS-500 Micro Air Sampler, Spectrex, Redwood City, CA, USA), which pulled air through the filter with a flow of 100 ml min^{-1} for 24 hours. As a control for ambient contaminants we collected scent from an empty glass cylinder using the same collection parameters. After sampling, the trapped volatile compounds were eluted with 50 µl of a hexane and acetone (9:1) mixture. All samples collected on the same day (4 to 9 plants) were pooled and stored in sealed glass vials at -30˚ C until GC-EAD analysis.

Chemical analysis

For analysis of headspace samples, gas chromatography with mass selective detection (GC-MSD) with thermodesorption was used. Samples (Tenax tubes)were injected into a GC (Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA) using a Gerstel thermodesorption system (TDS3, Gerstel, Mühlheim an der Ruhr, Germany) with a cold injection system (CIS4, Gerstel, Mülheim an der Ruhr, Germany). For

thermodesorption, the TDS was heated from 30˚C to 240˚C at a rate of 60˚C min^{-1} and held at the final temperature for 1 min. The CIS was set to −150˚C during trapping of eluting compounds from the TDS. For injection, the CIS was heated to 150˚C at a rate of 16˚C s^{-1} and then to 250˚C at a rate of 12˚C s^{-1}, the final temperature was held for 3 min. The GC oven was programmed to rise from a starting temperature of 50˚C (1 min hold) to 250˚C at a rate of 10˚C min^{-1}. The GC was equipped with a HP-5 column (0.25 mm diameter, 0.25 µm film thickness, 15 m length) and helium was used as carrier gas at a flow rate of 2 ml min^{-1}.

For analysis of nectar sugar derivatives liquid injection was used. Samples were injected into a GC (Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA) with an inlet temperature of 250˚C. The GC oven was programmed to rise from a starting temperature of 65˚C (2 min hold) to 300˚C at a rate of 6˚C min^{-1}. The GC was equipped with a HP-5 column 0.25 mm diameter, 0.25 µm film thickness, 15 m length) and helium was used as carrier gas at a flow rate of 2 ml min^{-1}.

Compound identification and quantification was conducted using a mass selective detector (Agilent MSD 5975, Agilent Technologies, Palo Alto, CA, USA) and ChemStation Enhanced Data Analysis program (version E.02.02) as described in (Schiestl et al. in press). Compounds were tentatively identified by comparison of spectra obtained from the samples, with those from a reference library (NIST ‘05 library). For further identification and quantification, synthetic standards of all compounds were run in two different concentrations (total injected amount: 10 and 100 ng) on the GC-MSD system to obtain a calibration curve for three to four compound-specific qualifier ions used for calculation of absolute amounts in the samples using the quantitation function in Agilent Chemstation. In addition, information about retention time and total mass spectrum of synthetic standards were used for final identification of compounds.

Electrophysiology

Gas chromatographic analysis with electro-antennographic detection (GC-EAD; Schiestl & Marion-Poll 2002) of headspace samples was performed using a gas chromatograph (Agilent 6890 N, Agilent Technologies, Palo Alto, CA, USA) equipped with a heated outlet for electroantennographic recordings (Effluent Conditioning Assembly , Syntech, Hilversum, the Netherlands). Antennal responses of *B. terrestris* workers were measured via EAD. For EAD recordings of *B. terrestris*, the tip of the excised antenna was abscised and the antenna was mounted between two glass capillaries filled with Ringer solution mounted on a micro-manipulator (Micro Manipulator MP-12, Syntech, Hilversum, the Netherlands). The electrode at the base of the antenna was grounded via an Ag/AgCl wire and the electrode at the distal end of the antenna was connected via a signal interface box (Syntech, Hilversum, the Netherlands) to a personal computer. Up to 5 µL of the headspace samples (eluates of Poropak filter) were injected splitless at 50˚C (1 min) into the GC followed by heating to 300˚C with a rate of 10˚C min^{-1}. The GC was equipped with an HP-5 column (0.32 mm diameter, 0.25 µm film thickness, 30 m length) and a flame ionization detector (FID). Hydrogen was used as carrier gas. A GC effluent splitter (Agilent G2855 Deans Switching System, Agilent Technologies, Palo Alto, CA, USA) was used to direct 50 % of the eluate, which was admixed to a purified and humidified air stream, over the excised antenna. EAD signals and FID responses were simultaneously recorded using Syntech software. Compounds releasing EAD responses were identified by comparison of retention times of samples with those of synthetic standard compounds.

Behavioral experiments with real flowers

Visual vs. olfactory signals

To assess the relative importance of visual and olfactory signals we conducted a dual-choice bioassay using glass cylinders (15 cm diameter, 29 cm height) as described in Burger et al. (2010). The cylinder used to present visual cues only was made of Quartz glass because of its UV transparency. The cylinder used for presenting olfactory cues only was made of borosilicate glass and was capped with four layers of green wire mesh (1 mm mesh size, sprayed with green, Dupli Color Products Group, Ohio, USA) to prevent bumble bees from seeing the yellow flowers. This cylinder had 20 slits (2 mm x 35 mm, between 12.5 cm and 25.5 cm of cylinder height) and was connected to a membrane pump (air pump 400, Eheim, Deizisau, Germany) to generate an outward air flow of 0.5 l min^{-1}. During the dual-choice bioassays, we placed four inflorescence spikes from *B. rapa* plants (from 8 plants in total) in each cylinder. Both cylinders were covered with a metal plate at the base to prevent the release of volatiles from the cylinder basis. The two glass cylinders were presented to free flying pollinators with a distance of 12 cm in the centre of the flight cage in which bumble bees were kept. One experimental run lasted 30 min, whereby the position of the two cylinders was exchanged after 15 min. After an experimental run, inflorescence spikes were replaced. We recorded every landing and caught and marked each landed individual to assure that an individual bumble bee was not counted twice. Because bumble bees couldn't land on the Quartz glass cylinder due to its smooth surface we counted each contact with the cylinder as a positive response (landing). The experiment was carried out with one hive. Before the onset of the experiment, bees were allowed to visit several flowering *B. rapa* plants.

Behavioral experiments with artificial flowers

Bioassay 1

Artificial flowers were constructed from 300 µl PCR tubes with the cap removed. The artificial corolla was made of a yellow paper disc (14 mm diameter; Sonnengelb, Artotz, Lenzburg, Switzerland) with a hole (6 mm diameter) in the centre which was placed over the tube. Septa (5 mm diameter, GR-2 Septa, Supleco, Bellefonte, PA, USA) were either soaked in a solution of 3 µl ml^{-1} phenylacetaldehyde in dichloromethane (Sigma-Aldrich, Buchs, Switzerland), or in a solution containing all other EAD-active compounds: 24.5 nl ml^{-1} acetophenone (Givaudan, Dübendorf, Switzerland), 27 nl ml^{-1} p-anisaldehyde (Sigma-Aldrich, Buchs, Switzerland) and 492 nl ml^{-1} α-farnesene (Sigma-Aldrich, Buchs, Switzerland) in dichloromethane. For emission rates see Table S1.

Bioassay 2

Artificial flowers were constructed from 2 ml Eppendorf tubes with the cap removed. The artificial corolla was made of a yellow paper disc (20 mm diameter; Sonnengelb, Artotz, Lenzburg, Switzerland) with a hole (9 mm diameter) in the centre which was placed over the tube. For the dual-choice bioassays conducted before and after the learning phases, one septum (3 mm diameter, GR-2 Septa, Supleco, Bellefonte, PA, USA) was soaked in a solution of 3 µl ml^{-1} phenylacetaldehyde in dichloromethane, the other in a solution of 60 nl ml^{-1} p-anisaldehyde. For compound concentrations in scent solutions used in the learning phase and according emission rates of septa see Table S2.

RESULTS

Behavioral experiments with real flowers

Visual vs. olfactory signals

Bumble bee workers (*Bombus terrestris*) significantly preferred olfactory signals over visual signals (Figure S1).

TABLES AND FIGURES

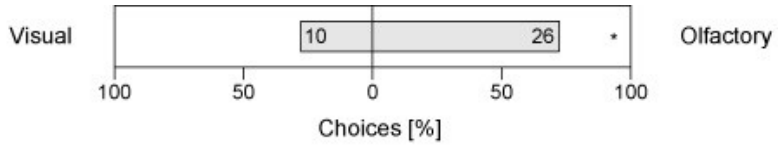


Figure S1 Dual-choice bioassays testing olfactory against visual floral signals using glass cylinders. The graphs show percentages of landings by *Bombus terrestris* on cylinders. The numbers in the bars are the absolute numbers of landings. Bumble bees significantly prefer olfactory over visual signals (Binomial test: n.s.: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).

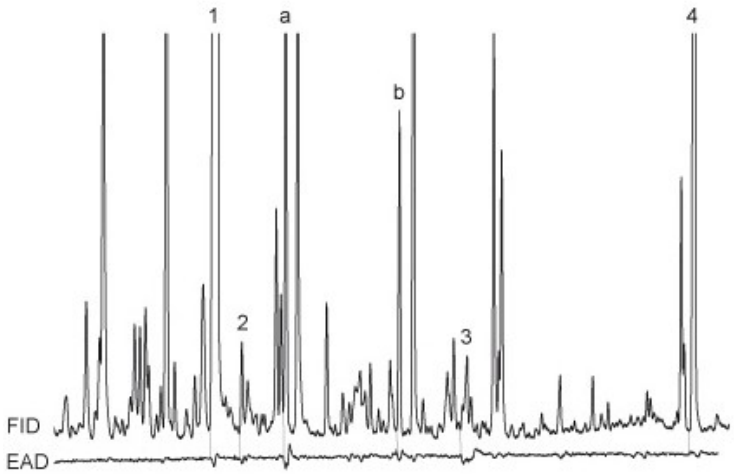


Figure S2 Gas chromatographic analysis of *Brassica rapa* floral scent with electroantennographic detection (GC-EAD) using antennae of *Bombus terrestris* workers. Peak numbers correspond to compounds (Table S3) that elicit electroantennographic responses. The two compounds nonanal (a) and decanal (b) were discarded from the dataset because the amounts in control samples collected from empty cylinders didn't differ from amounts collected from inflorescences.

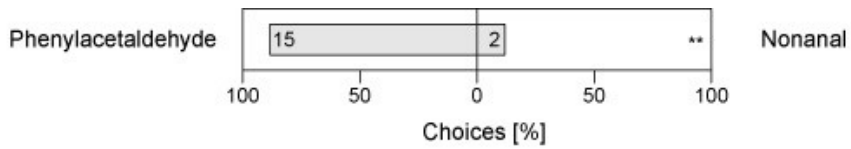


Figure S4 Dual-choice bioassays testing phenylacetaldehyde against nonanal in bumble bees previously exposed to *Brassica rapa* plants. Scent compounds were emitted from artificial flowers in about the amount of the mean + 2σ of *B. rapa* plants (data not shown). The graph shows percentages of landings by *Bombus terrestris* on artificial flowers. The numbers in the bars are the absolute numbers of landings. Bumble bees preferred phenylacetaldehyde over nonanal (Binomial test: n.s.: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).

Figure S5 Pictures of a *Brassica rapa* flower taken with a camera (D22, Nikon, Tokyo, Japan) equipped with a UV Nikkor 105 mm lens (Nikon, Tokyo, Japan) and UV-Filter (Baader U-Filter 60nm HBW/320-380nm, fully blocked VIS & IR, Baader Planetarium GmbH, Mammendorf, Germany).

Table S1 Amount of EAD-active compounds (ng l^{-1}) collected from whole inflorescences and septa. Scent was collected from twenty flowering plants and five septa prepared as described in the main text. Scent was collected in the early afternoon for 60 min.

Compound	Inflorescence		Septa
	Mean ± s.e.m.	Mean + 2σ	Mean ± s.e.m.
Phenylacetaldehyde	42 ± 9	126	140 ± 2
Acetophenone	0.81 ± 0.07	1.44	2.0 ± 0.2
p-Anisaldehyde	2.2 ± 0.4	5.6	6.5 ± 0.7
α-Farnesene	5.8 ± 0.9	14.3	18.4 ± 1.9

Table S2 Concentrations of phenylacetaldehyde and p-anisaldehyde in scent solutions (nl ml^{-1}) and corresponding amounts of these compounds (ng l^{-1}) collected from two septa per solution (numbers in the table are the means). Scent was collected in the early afternoon for 60 min.

	Phenylacetaldehyde honest	
Concentration of Biogluc	Phenylacetaldehyde	p-Anisaldehyde
0 %	0	13.3
33 %	69	13.3
66 %	114	13.3
100 %	160	13.3

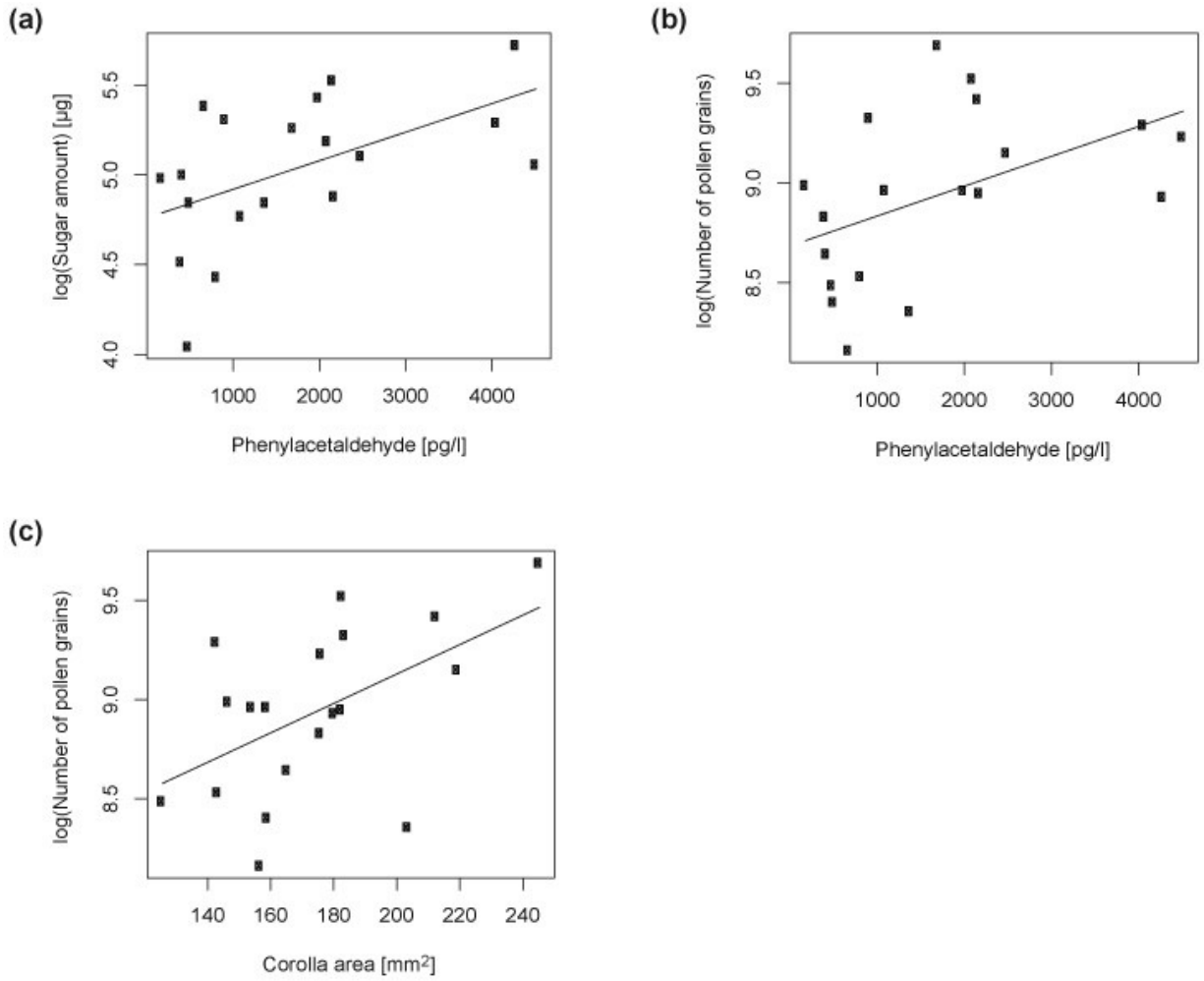
	p-Anisaldehyde honest	
	p-Anisaldehyde	Phenylacetaldehyde
0 %	0.0	160
33 %	5.5	160
66 %	9.2	160
100 %	13.3	160

Table S3 Number of individuals of *Bombus terrestris* workers responding to scent compounds in *Brassica rapa* inflorescence samples in coupled gas chromatography-electroantennographic detection (GC-EAD).

	Compound	*B. terrestris*	
No*	Name	Hive A (n = 6)	Hive B (n = 5)
	Aromatic compounds		
1	Phenylacetaldehyde	6	5
2	Acetophenone	6	3
3	p-Anisaldehyde	5	3
	Terpenoids		
4	α-Farnesene	3	1

* Compound number refers to Figure S1.

Figure S3 Linear regressions between total amounts of reward and (a-b) amount of phenylacetaldehyde, (c) corolla area measured per flower.



REFERENCES

Burger, H., Dotterl, S. & Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Functional Ecology, 24, 1234-1240.

Huber, F.K., Kaiser, R., Sauter, W. & Schiestl, F.P. (2005). Floral scent emission and pollinator attraction in two species of Gymnadenia (Orchidaceae). Oecologia, 142, 564-575.

Schiestl, F.P., Kirk, H., Bigler, L., Cozzolino, S. & Desurmont, G.A. (2014). Herbivory and floral signaling: phenotypic plasticity and trade-offs between reproduction and indirect defense. New Phytologist.

Schiestl, F.P. & Marion-Poll, F. (2002). Detection of physiologically active flower volatiles using gas chromatography coupled with electroantennography. In: Analysis of taste and aroma (eds. Jackson, JE & Linskens, HF), pp. 173-198.

CHAPTER II

The evolution of honest floral signalling in plants

Knauer, A.C. and Schiestl, F.P.

Department of Systematic and Evolutionary Botany, Zollikerstrasse 107, University of Zurich, 8008 Zurich, Switzerland

ABSTRACT

In many communication systems, signal-receivers profit from honest signals that indicate the signaller's quality. Low quality signallers should thus profit from cheating by emitting high signals. Under such a conflict of interests between signallers and signal-receivers, the maintenance of honest signals presents a puzzle. Some plants, however, emit honest floral signals to advertise floral rewards to pollinators. In *Brassica rapa*, corolla size and the floral volatile phenylacetaldehyde are correlated with nectar amount. It is, however, not known if these signals are under a conflict of interest between plant and pollinator and how signal honesty is maintained. In an outdoor experiment we found that bees developed preferences for a certain signal only if it was honest. In honestly signalling populations, individual plants should thus profit from cheating by gaining high pollinator visitation but saving costs of reward production. Nevertheless we could not detect selection for cheating in a cage experiment with *B. rapa* plants and bumble bees. However, besides the positive pollinator-mediated selection on honest signals by visitation frequency, we found that bee behavior additionally imposed selection on floral nectar amounts. Flower visitation time by bees was correlated with nectar amount and affected the number of seeds that visited flowers developed. Further, honest floral signals were correlated with the maximal number of seeds per fruit and both traits increased after soil fertilization. Together, these results imply that individuals with low nutrient availability in flowers can only produce low values of honest floral signals and at the same time profit less from high nectar amounts than individuals with high signals due to their limited capacities to produce seeds. In *B. rapa*, honest signalling is thus maintained by signal-associated differential benefits of nectar production caused by a combination of pollinator-mediated selection and resource limitation.

INTRODUCTION

Why do low-quality individuals not emit high-quality signals to receive the favorable behavior by signal-receivers? Why do organisms signal honestly when interests between signallers and receivers conflict? The evolution and maintenance of honest signals has been a highly debated puzzle over the last 40 years which led to a large body of theoretical literature and mathematical models (reviewed in Szamado 2011; Grose 2011). In contrast, empirical studies investigating the maintenance of honesty in connection to the characteristics of the biological system are still scarce (Grose 2011; Kotiaho 2001; but see Møller and Delope 1994; Polnaszek and Stephens 2014). While in some systems the correlation between the signal and the signaller's quality are maintained by simple constraints, in other systems it represents an evolutionary stable strategy (ESS) established by natural selection (Szamado 2011). Without constraints, honest signalling should evolve when high-quality individuals gain a net benefit from producing high-quality signals while low-quality individuals do not gain a net benefit. Such net benefits can be prevented by higher costs and/or lower benefits of signal production in cheaters compared to honest signallers (Higham 2014). However, constraints, benefits and costs of signal production can strongly depend on the system under study.

Plants signal to their animal pollinators to advertise rewards and in return receive directed pollen transfer between individuals (Schiestl and Johnson 2013). Because rewards are normally concealed within flowers, pollinators rely on floral signals, like color, size and scent, when choosing plants for visitation. For low-quality individuals (with little reward) it should therefore be advantageous to emit high signals to still receive high visitation number by pollinators. Nevertheless, various plant species emit honest floral signals correlated with nectar or pollen amounts (Howell and Alarcon 2007; Stanton and Young 1994; Gómez et al. 2008; Pélabon et

al. 2012; Ida and Kudo 2010; Armbruster et al. 2005). Such honest floral signals could be maintained by several different mechanisms. First, signals can be so-called indices – signals that are intrinsically linked to the signaller's quality. Pleiotropy (genetic constraints) (Conner 2002) and signals emitted by rewards themselves (physiological constraints) (Dobson et al. 1999; Raguso 2004) could cause such intrinsic links between floral signals and rewards. Second, by mathematical modelling, Cohen and Shmida demonstrated that floral signal honesty can be an ESS (Cohen and Shmida 1993). According to their model honesty can be maintained by pollinator-mediated selection on floral signals and reward in combination with resource limitation in signal and reward production (by the same resource) and variable resource allocation to flowers within plant populations.

To be an ESS, pollinators must impose selection on floral rewards and signals. Pollinator-mediated selection on rewards requires sanctions by pollinators (Broom et al. 2013; Cohen and Shmida 1993): Once landed on inflorescences, pollinators can verify floral rewards by consuming it and adjust their behavior accordingly in subsequent interactions with the plant. Revisitation, the number of flowers that pollinators visit and probing time per flower can be subject to such behavioral adjustments and select for increased floral rewards (Kadmon and Shmida 1992; Cresswell 1999; Mitchell 1993; Galen and Plowright 1985; Brandenburg et al. 2012; Real and Rathcke 1991; Makino and Sakai 2007). Pollinator-mediated selection on floral signals, in contrast, requires the pollinator's preference for high or low signal values. While in specialist pollinators, preferences for honest signals could be innate, in generalist pollinators they must be learned as different plant species may emit different honest signals. This learning process requires the pollinator's capability to identify honest floral signals and to develop preferences for them. Indeed, in learning assays, bumble bees distinguished between honest and

dishonest floral signals and developed a preference for honest floral signals in rather short time (Knauer and Schiestl 2015). But these cage experiments were conducted with only one type of artificial flowers that emitted two floral signals. In natural environments however, pollinators normally experience many different plant species which emit various signals each. Although the use of honest signals should be advantageous for pollinators as it improves foraging efficiency, the recognition and remembering of honest floral signals represents an enormous challenge to pollinator's learning capacities.

Although honest signalling has been documented for several plant species, the mechanisms maintaining honesty have barely been investigated. In *Brassica rapa* two floral signals honestly indicate nectar amount: corolla size and the floral volatile phenylacetaldehyde (Knauer and Schiestl 2015). The mechanism maintaining signal-reward correlation in *B. rapa* is still unknown, however. As nectar of this species does not emit any scent, a physiological constraint can be excluded as the cause of honesty (Knauer and Schiestl 2015). Also, pleiotropy is an improbable source of signal-nectar correlations as petal development, the synthesis of phenylacetaldehyde and nectar production involve supposedly different metabolic pathways (Borghi et al. 2017). However, honesty might be an ESS maintained through pollinator-mediated selection on honest signals and rewards in combination with resource limitation in both traits. In fact, in cage experiments, both honest floral signals attracted pollinators and were under positive pollinator-mediated selection (Knauer and Schiestl 2017). However, it is still unclear if wild pollinators that forage in natural environments show the same preferences, and if these preferences are caused by signal honesty (but see Armbruster et al. 2005). Further, neither pollinator mediated selection on floral reward through sanctions, nor resource limitation in signal and reward production have been measured in *B. rapa*. Thus, the aim of this

study was to identify the mechanism maintaining signal-reward correlation in *B. rapa*. Specifically, we aimed to answer the following questions: 1) Do pollinators in natural environments only develop preferences for floral signals when they are honest? 2) Is the signal-nectar correlation under selection? 3) Do pollinators select for high signal values by increased visitation frequency? 4) Do pollinators select for large nectar amounts by sanctions? 5) Are signals and nectar limited by the same resources?

METHODS

Study system

Brassica rapa is a self-incompatible, annual or biennial herb native to Eurasia (Watanabe et al. 2000). It has a generalized pollination system with a wide variety of pollinators from the orders Hymenoptera, Diptera, Lepidoptera and Coleoptera (Rader et al. 2009). However, especially different bee species are important pollinators in terms of visitation rate and pollination efficiency (Sahli and Conner 2007; Rader et al. 2009).

B. rapa seeds were collected in a natural population from about 100 individuals (population size over 1000 individuals, Maarssen, the Netherland) and grown under standardized light, soil and watering conditions in a greenhouse. All plants were treated every second week with the pesticides Kendo and Thiovit (Maag, Dielsdorf, Switzerland) until start of flowering. The bumble bee (*Bombus Terrestris*) colonies used in this study were purchased from Andermatt Biocontrol (Andermatt, Switzerland) and the hives were kept in a flight cage (3 x 1 x 1 m). For each experiment (measurement of pollinator attraction/selection and test for sanctions) only one hive was used. Bumble bees were fed on pollen (purchased directly from beekeepers) and sugar solution (Apiinvert, Südzucker AG, Ochsenfurt). Additionally

we exposed the bees to 20 to 30 flowering *B. rapa* plants for at least 3 hours before experimental use.

Use of honest signals by bees in a natural environment – Experiment 1

This experiment was conducted to measure the bee's preference for honest floral signals compared to dishonest ones in a natural foraging environment. We placed 28 *B. rapa* plants on a meadow in the botanical garden of Zurich for during 6 days, for 3.5 h each. During this time wild pollinators were allowed to visit flowers and learn to associate signals with rewards. Each day plants were assigned to one of the following learning treatments: a) no scent manipulation; plants emitting phenylacetaldehyde as an honest signal; b) scent manipulation; plants emitting different amounts of phenylacetaldehyde randomly as a dishonest signal (see below for description of scent manipulation). These two treatments were alternated between days, three times each, during sunny and warm days in September 2013. After the 3.5 h that plants were exposed to pollinators, they were removed and immediately replaced with five test-plots of four plants each. These plants were used to test the bees' preference for phenylacetaldehyde after the different learning treatments. We augmented phenylacetaldehyde emission in two plants per plot whereas the other two plants emitted natural amounts of the compound (no manipulation). Throughout the 30 min that plots were maintained, bee visits to plants with high and low phenylacetaldehyde emission were recorded continuously. Although *B. rapa* plants were also visited by syrphid flies, we focused on bees as they represent the main pollinators (Rader et al. 2009). Within each plot, plants with the same scent treatment were placed side by side with a distance of 0.5 m; the distance between plots was 2 m. Further, to control for genetic background, we used representatives of the same full sib families at the same positions per plot on subsequent experimental days (after

different learning treatments). Also, flower number did not differ between the two scent treatments for any experimental day (data not shown).

We augmented the emission rate of phenyacetaldehyde using scent application by grey rubber septa (Supleco, Bellefonte, PA, USA) (Huber et al. 2005). Rubber septa were soaked in solutions of phenylacetaldehyde in dichloromethane for one hour and afterwards dried for five hours before experimental use to obtain stable emission rates. For the learning treatment b) we used four different solutions (0, 1, 2 and 3 µl ml^{-1} phenylacetaldehyde in dichloromethane, 7 septa per solution). For the scent augmentation in plots we used a solution of 3 µl ml^{-1} phenylacetaldehyde in dichloromethane. Septa prepared in this way emitted phenylacetaldehyde in a concentration of about the *mean + 2σ* of *B. rapa* inflorescences (see Knauer and Schiestl 2015 for more details). Control septa were soaked in pure dichloromethane.

To analyse the effect of the phenylacetaldehyde augmentation on bee attraction in plots, we used a generalized mixed effect model with a poisson distribution, the number of visits as the response and scent augmentation and flower number as explanatory variables. Day and plot were included as random effects in the model. This analysis was done separately for the two learning treatments. Additionally, we fitted a model with all data to test for differences in the effect of scent augmentation after different learning treatments. Again, a generalized mixed model was used, but the learning treatment was included as an explanatory variable to test for a significant learning treatment x scent augmentation interaction.

Selection on signals, reward and signal honesty – Experiment 2

To test for selection on honest floral signals and reward as well as the degree of honesty between the two, we conducted a plot experiment exposing 36 *B. rapa* plants (6 x 6, 40 cm distance between plants) to 12 bumble bees in an outdoor cage

(3 x 3 x 2 m). For all plants we measured corolla size, floral scent and nectar volume (measurement of floral traits described below) one day before pollinator exposure. Additionally, total number of flowers that opened during pollinator exposure were counted (see also Knauer and Schiestl 2017). Each plot was kept in the cage for two subsequent sunny days, every day bumble bees were released to visit plants twice (about 15 min, 3 bees at the time). During pollinator exposure all visits to inflorescences were recorded. Afterwards all bees were marked with a dot to avoid multiple usages of individuals (pseudoreplication). In total we conducted three replicates leading to a sample size of 108 plants and 36 bees in total.

To complete fruit development, plants were continuously watered for at least another three weeks after pollinator exposure. Afterwards, the number of seeds per individual was counted. Number of seeds represents an accurate estimate of the lifetime female fitness since *B. rapa* is annual and reproduces only once during its life. Relative fitness was estimated by dividing the seed set of each individual by the replicate mean.

Before statistical analysis, the number of seeds was ln(1+x) transformed and phenylacetaldehyde was BoxCox-transformed (with $\lambda = 0.2$) to obtain homogeneity of variance and approach normal distributions of residuals. Standardization of floral traits by z-transformation was done within replicates to eliminate differences in means and variances. To test for selection on floral signals, reward and signal-reward correlations we fitted a model with the fitness as response and the nectar amount, corolla size and phenylacetaldehyde emission as explanatory variables. Additionally we included the two interaction terms between the nectar amount and each floral signal to test for correlational selection. Significant correlational selection represents selection on signal honesty. The number of flowers was included into the model to control for display size.

Further, to test for the effect of floral signals on primary pollinator attraction, we fitted a model with number of visits by bumble bees as response and corolla size, phenylacetaldehyde and flower number as explanatory variables. Nectar amount was not included into the model as returning visits could be neglected in this experiment because individual bumble bees were released to forage on plots for very short time.

Sanctions by pollinators – Experiment 3

This experiment was conducted to investigate the potential for sanctions against cheaters imposed by bumble bees. We placed 36 *B. rapa* plants (6 x 6, 40 cm distance between plants) in a cage (3 x 3 x 2 m) and released 5 bumble bees for 15 minutes. One day before the experiment we measured floral nectar volume, corolla size and phenylacetaldehyde emission (measurement of floral traits described below) for all plants. During pollinator exposure we recorded the number of flowers that bumble bees visited and the time they spent on the inflorescence for each visited plant. The mean time spent per flower was calculated as *(total time on inflorescence)/(number of visited flowers)*. To analyse data we used multiple regressions with the nectar volume, corolla size and phenylacetaldehyde as explanatory variables. The visitation time per flower and the percentage of visited flowers were fitted as responses in two separate models. For statistical analysis phenylacetaldehyde was BoxCox-transformed (with $\lambda = 0.2$) to obtain homogeneity of variance, and approach normal distributions of residuals.

Fitness effects of number of visits and flower visitation time – Experiment 4

Number of visits

Because corolla size and phenylacetaldehyde emission positively affected bumble bee attraction in *B. rapa* plants, we measured the effect of the number of visits on

plant fitness. Plant individuals were exposed to 1,2 or 3 bumble bees successively in a netting (3 x 1 x 1 m). For each number of visits we measured 9 plants (27 plants in total) and each bumble bee was only used once in the experiment (54 bumble bees). We let each bumble bee first visit a pollen donor plant (a different plant for each bumble bee) before it was allowed to visit the target plant. The visit was considered as completed when bumble bees moved on to a third plant in the cage. For each experimental plant we counted the number of open flowers and, after fruit development, the total number of fruits and seeds. Finally, data was analysed by a multivariate linear regression with the number of fruits as response and the number of visits and the flower number as explanatory variables. Additionally we tested for an effect on seed development by fitting a regression with the number of seeds per fruit as response and the number of visits as explanatory variable.

Flower visitation time

Because we found a positive association between floral nectar volume and visitation time by bumble bees, we tested for a positive effect of visitation time on plant fitness. For that purpose we allowed 18 *B. rapa* plants to be visited by one bumble bee each (18 bumble bees in total) in a netting (3 x 1 x 1 m). The whole visit was recorded by a camera (Sony handycam HDR-CX220E) and the visitation time for each visited flower was measured. After fruit ripening we counted the number of seeds that visited flowers developed. Identification of flowers in videos was guaranteed by colored markings on inflorescences. Before bumble bees were released to visit the experimental plants we let them forage on two *B. rapa* plants to ensure substantial pollen load for pollination. Finally, data was analysed by a mixed effect model with the number of seeds as response, the visitation time as explanatory variable and the

plant individual as a random effect. The number of seeds was BoxCox-transformed (λ = 0.4) to obtain normal distribution of residuals.

Resource limitation – Experiment 5

To test for resource limitation by soil nutrients in honest floral signals and nectar we tested the response of these traits to the application of fertilizer. 36 *B. rapa* plants were grown in 400 ml of standardized soil (Einheitserde, Sinntal-Altengronau, Germany) in the greenhouse and assigned to the following two treatments alternately: 1) application of 0.5 g fertilizer; 2) control (no fertilization). We used long term fertilizer (Osmocote Exacte Standard 3-4 month, 16% nitrogen, 9% phosphate, 12% potash, 1.2% magnesium), which is solid and releases nutrients for 6 month at constant rate. Therefore, application of fertilizer was done only once when inflorescences started developing (6 weeks after sawing). When plants were in full flower we measured corolla size, phenylacetaldehyde emission and nectar amount (measurement of floral traits described below). Further, we quantified the total number of flowers for each of these plants and hand-pollinated 9 flowers per individual with pollen from 3 donor plants (3 flowers per pollen donor) to calculate mean number of seeds per fruit after fruit ripening. Incompatibility reactions were identified as specific crossings not producing fruits (one out of three pollen donors did not produce seeds). Finally, to test for differences in floral traits between fertilization treatments we used t-tests (a separate test was fitted for each trait). Phenylacetaldehyde was BoxCox-transformed (λ = 0.2) to obtain normal distribution.

Association between honest floral signals and number of seeds - Experiment 6

Because we did not find resource limitation in nectar production, but instead in the number of seeds per fruit, we tested for an association of this fitness measure with honest floral signals. Such an association would indicate differential benefits of nectar volume depending on signal value, as nectar volume positively affected the number of seeds through increased visitation times by bumble bees. According to mathematical models, such differential benefits can maintain signal honesty (Grafen 1990; Johnstone 1997). We grew 29 plants under standardized conditions and measured corolla size and phenylacetaldehyde emission when plants were in full bloom (measurement of floral traits described below). Further, we quantified the maximal number of seeds per fruit by hand-pollinating 9 flowers per individual with pollen from 3 donor plants (3 flowers per pollen donor). After fruit ripening, the mean number of seeds per fruit was quantified. Incompatibility reactions were identified as specific crossings not producing fruits (one out of three pollen donors did not produce seeds). Finally, we calculated Pearsons's product-moment correlation for the mean number of seeds per flower and honest signals.

Measurement of floral traits

For scent collection from inflorescences we used the push-pull headspace collection method (Tholl et al. 2006; Schiestl et al. 2014). Inflorescences were enclosed in glass cylinders (dimensions: 5 cm diameter, 25 cm height; all glass cylinders were treated previously with sigmacoate (Sigma-Aldrich, Buchs, Switzerland)). The bottom of the cylinder was closed with a teflon plate with a central hole allowing for the insertion of the peduncle without injuring it. For volatile collection glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into a small opening in the cylinder and attached to a vacuum pump

(DC06/04/20F, Fürgut GmbH, D-88459 Tannheim) with a silicon tube. Air was pulled through the Tenax tubes at a flow rate of 150 ml min^{-1}. After passing the tube, the air was circulated back (with the same flow rate) to the glass cylinder through another Tenax tube (Tenax GR 60/80, Scientific Instrument Services, Old York, NJ, USA), which was inserted through a second opening, to clean the incoming air. The number of flowers inside the cylinder was counted to calculate volatile amounts per flower. All collections took place between 11^{00} and 15^{00}hrs in the greenhouse under standardized temperature, humidity and light conditions. After scent collection the Tenax tubes were stored at -30˚C until chemical analysis.

For the analysis of floral volatiles, gas chromatography with mass selective detection (GC-MSD) was used. Samples were injected into a GC (Agilent 6890 N; Agilent Technologies, Santa Clara, CA, USA) using a Gerstel thermodesorption system (TDS3; Gerstel, Mülheim, Germany) with cold injection (KAS4; Gerstel). The GC was equipped with a DB-5 column (0.32 mm ID, 0.25 lm film thickness, 30 m length), and helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Compound determination was done by comparing the spectra obtained from the natural samples with those of synthetic standard compounds. Standard compounds were also used for compound quantification using dose response curves for each volatile (Schiestl et al. 2014).

Petal length and width were measured in three fully opened flowers per individual. Means of petal length and width were used to estimate the corolla size per flower as $\pi*length*width$.

Nectar was collected from three flowers per individual with 5 µL micropipettes (Blaubrand, Wertheim, Germany). Nectar volume was then calculated from the nectar-filled length in the micropipette. In *B. rapa* nectar volume is highly correlated

to nectar sugar amounts ($R = 0.75$, $P < 0.001$, see also Knauer and Schiestl (2015)) and therefore a good estimate of reward amount.

RESULTS

Use of honest signals by bees in a natural environment – Experiment 1

We tested bees for their preference for phenylacetaldehyde, when it was emitted either as an honest or a dishonest signal. In total we observed 235 visits by bees: 220 by *Apis mellifera* and 15 by *Bombus sp*.. After bees had visited unmanipulated plants emitting phenylacetaldehyde as an honest signal (leaning treatment a), they showed a preference for high phenylacetaldehyde emission (estimate ± s.e. = 0.50 ± 0.19, $z = 2.609$, $P = 0.004$). Plants with augmented phenylacetaldehyde emission received in total 1.7 times more visits by bees than unmanipulated plants (Figure 1). In contrast, after bees had visited manipulated plants emitting phenylacetaldehyde as a dishonest signal (learning treatment b), bees did not show any preference for phenylacetaldehyde (estimate ± s.e. = -0.26 ± 0.18, $z = -1.388$, $P = 0.17$) (Figure 1). Accordingly, the learning treatment had a significant effect on the bee attraction by scent augmentation (significant learning treatment x scent augmentation interaction: estimate ± s.e. = 0.77 ± 0.26, $z = 2.894$, $P = 0.003$).

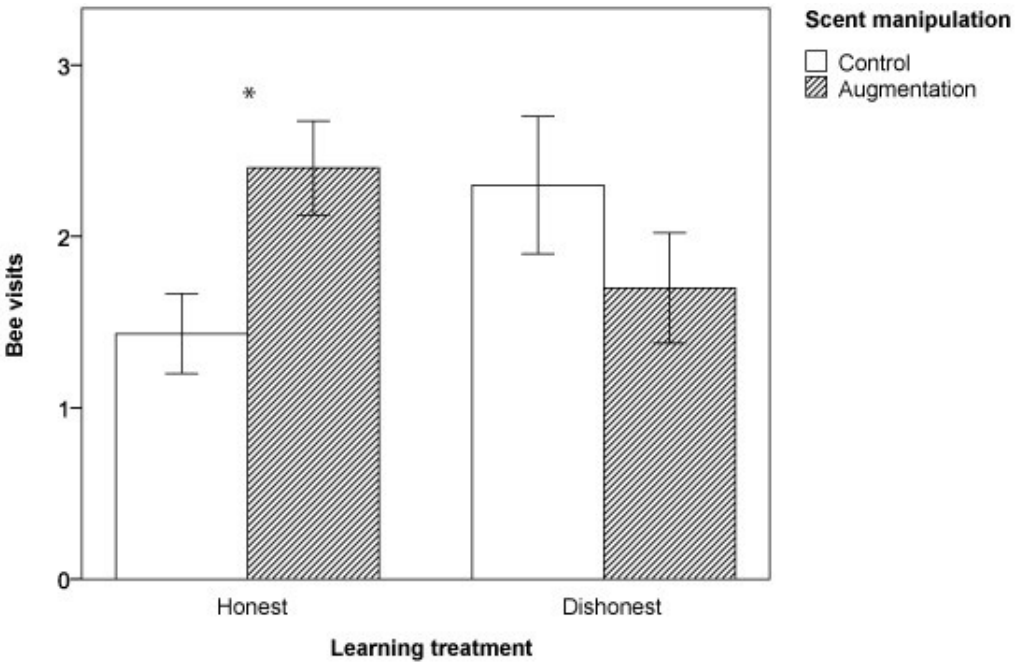


Figure 1 Number of Bee visits to plants with natural and augmented emission of the floral volatile phenylacetaldehyde after bees had been foraging on plants emitting phenylacetaldehyde as an honest signal (learning treatment "honest") or as a dishonest signal (learning treatment "dishonest"). Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk (N = 30 per treatment).

Selection on signals, reward and signal honesty – Experiment 2

We tested for selection on honest floral signals, nectar amount and the signal-nectar correlation in a cage experiment. Both corolla size and phenylacetaldehyde were significantly correlated with nectar volume (corolla size: $R = 0.33$, $P < 0.001$; phenylacetaldehyde: $R = 0.49$, $P < 0.001$). Further, we found positive directional selection on corolla size, phenylacetaldehyde and nectar volume (Table 1). However, no correlational selection on the reward-signal association could be detected, neither for corolla size nor for phenylacetaldehyde (Table 1). Further, corolla size as well as the amount of phenylacetaldehyde were positively associated with the number of first visits to inflorescences by bumble bees (Table 2).

Table 1 Directional selection gradients on floral traits and signal honesty (N = 108 plants). Selection gradients were calculated by a multiple regression with relative seed set as fitness estimate and standardized floral traits as explanatory variables. To test for selection on honesty the signal x reward interactions were included.

Floral trait	β ± s.e.	t	P
Nectar volume	0.13 ± 0.06	2.138	**0.035**
Corolla size	0.20 ± 0.06	3.596	**< 0.001**
Phenylacetaldehyde	0.13 ± 0.06	2.200	**0.030**
Corolla size x nectar volume	-0.04 ± 0.06	-0.735	0.46
Phenylacetaldehyde x nectar volume	-0.004 ± 0.06	-0.082	0.93
Flower number	0.20 ± 0.05	3.912	**< 0.001**

Significant results are given in bold

Table 2 Effect of honest floral signals on the number of first visits by bumble bees (N = 108 plants). To calculate pollinator attraction we used a multiple regression with number of visits to inflorescences as response and standardized floral traits (honest signals) as explanatory variables.

Floral trait	β ± s.e.	z	P
Corolla size	0.20 ± 0.07	2.870	**0.004**
Phenylacetaldehyde	0.18 ± 0.07	2.558	**0.011**
Flower number	0.36 ± 0.06	5.829	**< 0.001**

Significant results are given in bold

Sanctions by pollinators – Experiment 3

To test for sanctions against cheaters by bumble bees we analysed how floral reward affects their foraging behavior after landing on inflorescences. Bumble bees spent significantly more time on flowers with larger nectar amounts (estimate ± s.e. = 10.2 ± 2.7, t = 3.773, P = 0.003) (Figure 2). In contrast, corolla size did not affect floral visitation time (t = 1.159, P = 0.27) and phenylacetaldehyde even significantly decreased the time (estimate ± s.e. = -0.22 ± 0.06, t = -3.552, P = 0.005). Further, neither nectar amount nor corolla size or phenylacetaldehyde had an effect on the percentage of flowers that bumble bees visited in an inflorescence (nectar amount: t

= 0.629, P = 0.54; corolla size: t = 0.223, P = 0.83 ; phenylacetaldehyde: t = -2.036, P = 0.07).

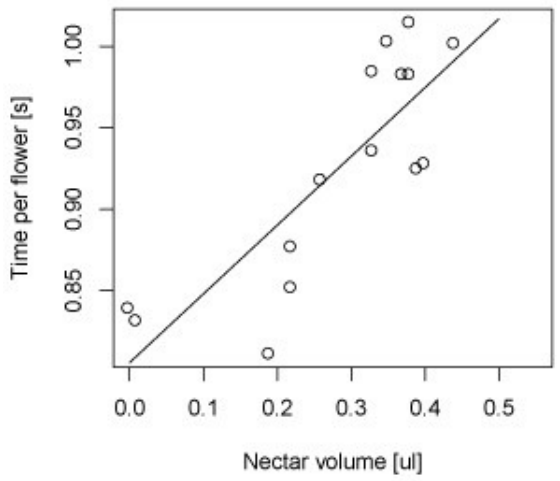


Figure 2 Association between bee visitation time and nectar amount (N = 15).

Fitness effects of number of visits and flower visitation time – Experiment 4

To test for the potential benefit of cheating and the consequences of sanctions by pollinators for plant fitness we analysed the effects of the number of visits and the flower visitation time by bumble bees on plant fitness. Both, number of visits and visitation time positively affected plant fitness. Each additional visits by bumble bees significantly increased the total number of fruits by 2.4 ± 1.1 (t = 2.131, P = 0.044). Interestingly, the number of seeds per fruit was not affected by the number of visits (t = 1.357, P = 0.19). In contrast, the visitation time per flower significantly increased the number of seeds that visited flowers developed (seeds per fruit) by 0.30 ± 0.05 s^{-1} (t = 5.853, P < 0.001).

Resource limitation – Experiment 5

A fertilization experiment was conducted to test for resource limitation in nectar amount and floral signals. Fertilization of *B. rapa* plants did not affect the amount of nectar per flower (t = 0.21, df = 28.70, P = 0.83). But it significantly increased the two honest signals phenylacetaldehyde (t = 2.10, df = 28.34, P = 0.044) and corolla size (t = 2.11, df = 25.91, P = 0.045). Also, fertilized plants had significantly more flowers (t = 2.69, df = 28.61, P = 0.012) and produced a higher number of seeds per fruit after hand-pollination (t = 3.66, df = 28.98, P = 0.001) (Figure 3).

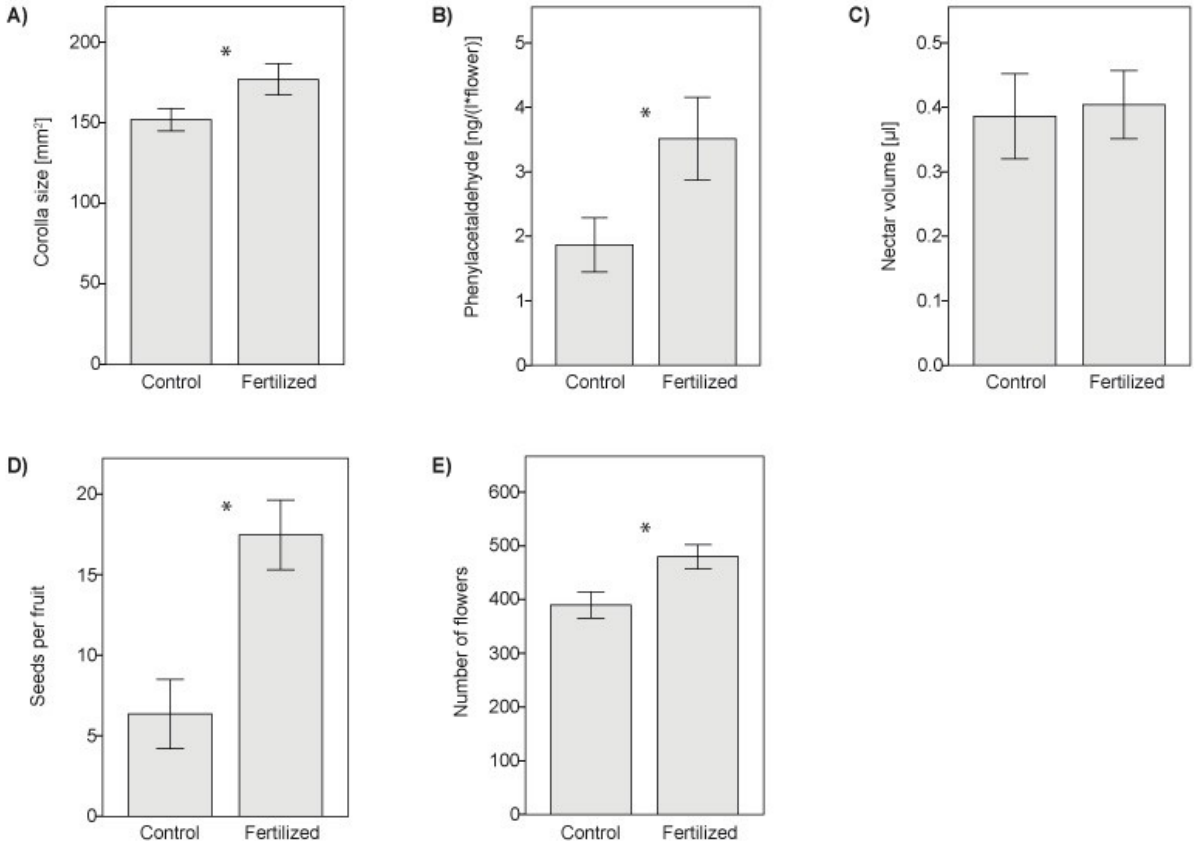


Figure 3 Effect of fertilization on floral traits. **A)** corolla size; **B)** emission of the floral volatile phenylacetaldehyde per flower; **C)** nectar volume per flower; **D)** the maximal number of seeds per fruit after hand-pollination; **E)** the total number of flowers per inflorescence. Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk (N = 18 per treatment).

Association between honest floral signals and number of seeds – Experiment 6

Because honest floral signals and seed development were limited by nutrient content in soil, we tested for a correlation between these traits indicating differential benefits of nectar volume depending on signal value. The maximal number of seeds that flowers developed after hand-pollination was significantly correlated with corolla size ($R = 0.43$, $t = 2.44$, $P = 0.022$) as well as the amount of phenylacetaldehyde that flowers emitted ($R = 0.41$, $t = 2.30$, $P = 0.029$) (Figure 4).

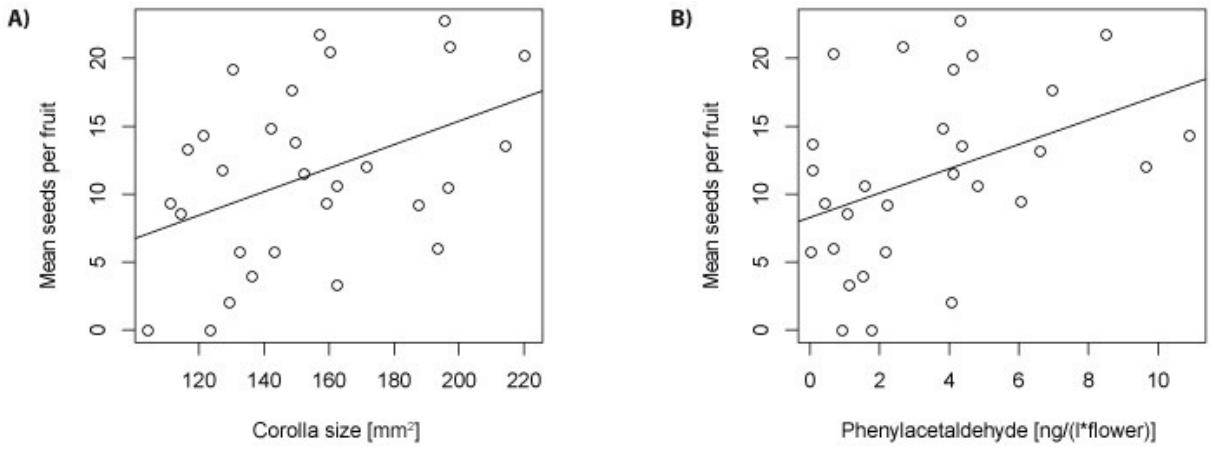


Figure 4 Correlation between the number of seeds per fruit after hand-pollination and honest floral signals. **A)** corolla size **B)** emission of the floral volatile phenylacetaldehyde per flower.

Mechanism maintaining honesty in *B. rapa*

Based on our results from experiment 1 – 5 we built a hypothesis of the mechanism that maintains honesty in *B. rapa.* This mechanism involves pollinator mediated selection on floral signals and nectar amount in combination with resource limitation in signals and seed development (Figure 5). We explain our hypothesis in detail in the discussion part.

DISCUSSION

Although honest signalling has been documented for various plant species (Armbruster et al. 2005; Pélabon et al. 2012; Gómez et al. 2008; Stanton and Young 1994; Knauer and Schiestl 2015), the mechanisms maintaining honest signalling in plant-pollinator communication are still poorly understood. Here we show that in honestly signalling plant populations, individuals benefit from high signal values by increased pollinator visitation rates irrespective of their reward value. This should lead to the breakup of honest signalling over time. Nevertheless, we found significant signal-reward correlations in *B. rapa* and no selection for cheating. We resolve this apparent contradiction by showing that honest signalling is maintained by a combination of pollinator mediated selection and nutrient limitation. Our paper shows that resource availability impacts signal evolution in plants, an aspect that has received little attention so far.

Although learning of honest signals has been demonstrated in cage experiments (Knauer and Schiestl 2015), its relevance in nature is still unknown. Here, we found that augmented phenylacetaldehyde emission increased bee attraction, but only when this compound was emitted as an honest signal in the plant population. This result demonstrates that generalist pollinators are able to identify and use a plant's honest floral signals also in natural habitats. Honesty might be lost by increasing reward depletion by pollinators. In plant species that open new flowers every day and/or have high nectar replenishment rates however, honesty should be present at the beginning of the main pollinator activity. Thus, pollinator preferences for honest floral signals imply a conflict of interest between plants and pollinators. While pollinators could profit from honest floral signals by receiving maximal reward amounts, for plants it should be advantageous to receive high pollinator visitation by

emitting high signal values even when reward amounts are low. This conflict raises the question of how signal honesty is maintained in *B. rapa*.

The mechanisms maintaining signal-reward correlations in flowers have so far barely been investigated. According to Cohen and Shmida's (1993) mathematical model, honest floral signalling can be maintained by pollinator-mediated selection on signals and reward in combination with limitation in these floral traits by the same resource and its variable allocation to flowers. In our experiment we indeed established pollinator-mediated selection on honest floral signals and nectar amounts, but only detected nutrient limitation in signals and not in rewards. Although this result is in line with several studies that measured very small costs of nectar production in plants (Rutter and Rausher 2004; Harder and Barrett 1992; Odowd 1980), nectar could still be associated with costs in terms of other resources such as water or light (Petanidou et al. 1999; Southwick 1984). However, in *B. rapa* signal-reward correlations can be explained by signal-associated differential benefits of nectar volume. Bumble bees visited flowers with high nectar amounts longer, which positively affected the number of seeds that flowers developed. The maximal number of seeds that flowers could develop, however, was correlated with honest floral signals - likely because both traits were limited by absolute nutrient allocation to flowers. Thus, low-signal plants benefit less from high nectar amounts than high-signal individuals due to their reduced capacity to develop seeds. According to general honest signalling models, honesty can indeed be maintained by lower signal benefits in low-quality individuals compared to high-quality individuals (differential benefits) (Johnstone 1997; Grafen 1990). In *B. rapa*, however, the differential benefits of nectar amounts are seemingly linked to signal emission metabolically (Figure 5).

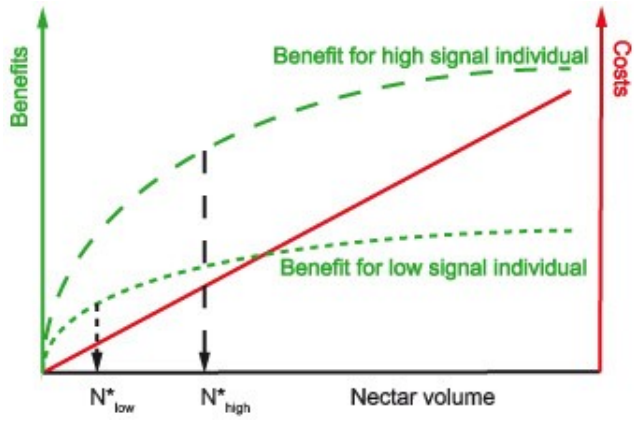


Figure 5 Hypothetical mechanism maintaining honest floral signalling in *B. rapa*: Honesty is maintained by differential benefits (seed number) of nectar volume that depend on floral signals. Individuals with low resource allocation to flowers have lower signals and gain a smaller benefit from a given nectar volume than individuals with high resource allocation to flowers. The optimal nectar volume for low signal individuals is N^*_{low}, for high signal individuals it is N^*_{high} (optima are defined by the maximal difference between benefits and costs). The graphic is adapted from Johnstone (1997).

Here we have shown that pollinator-mediated selection on floral signals and reward in combination with resource limitation in signal production and seed development can maintain honest signalling in plant-pollinator communication. This mechanism should provoke the evolution of honest floral signals in plant species that experience strong pollen limitation and are subject to sanctions by pollinators. Further, honesty should only evolve in resource limited signals and be supported by high variability in absolute resource allocation to flowers between individuals. Such absolute allocation may depend on the resource availability in the environment, the plant's capacity to assimilate them and the proportion that is allocated to flowers relative to other tissues. The relationship between resource availability and the variability in resource allocation may depend on the type of resource (Stanton et al. 2000). Low nutrient availability, however, has been shown to reduce phenotypic variability in *Sinapis arvensis* (Stanton et al. 2000), possibly because individuals with good nutrient assimilation and high allocation to flower are not capable to express

these traits. Accordingly, we predict to find honesty mainly and in habitats with intermediate to high nutrient availability and patchy resource distribution, factors that should modulate variation in nutrient limitation in plant populations. Also, honesty should evolve only in signals with high efficacy costs.

Our study provides first evidence that differential benefits, rather than differential costs maintain signal honesty (see also Møller and Delope 1994; Polnaszek and Stephens 2014). The here suggested mechanism maintaining floral honesty depends on the interaction between genetic plant traits and environmental factors. Honest floral signals should therefore only evolve in certain signals, plant species and environments. Future investigations should incorporate those aspects to deepen our understanding of signal evolution in various plants and habitats.

ACKNOWLEDGEMENT

We would like to thank Rayko Jonas and Markus Meierhofer for their help with plant cultivation. Also, we thank Franz Huber for his support in the GC lab and Alice Balmer and Daniel Gervasi for their help with experiment implementation. The research leading to these results has received funding from the European Union's Seventh Framework Program ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement n° 281093.

REFERENCES

Armbruster WS, Antonsen L, Pelabon C (2005) Phenotypic selection on Dalechampia blossoms: Honest signaling affects pollination success. Ecology 86 (12):3323-3333. doi:10.1890/04-1873

Borghi M, Fernie AR, Schiestl FP, Bouwmeester HJ (2017) The Sexual Advantage of Looking, Smelling, and Tasting Good: The Metabolic Network that Produces Signals for Pollinators. Trends Plant Sci 22 (4):338-350. doi:10.1016/j.tplants.2016.12.009

Brandenburg A, Kuhlemeier C, Bshary R (2012) Hawkmoth Pollinators Decrease Seed Set of a Low-Nectar Petunia axillaris Line through Reduced Probing Time. Current Biology 22 (17):1635-1639. doi:10.1016/j.cub.2012.06.058

Broom M, Ruxton GD, Schaefer HM (2013) Signal verification can promote reliable signalling. Proceedings of the Royal Society B-Biological Sciences 280 (1771). doi:2013156010.1098/rspb.2013.1560

Cohen D, Shmida A (1993) The evolution of flower display and reward Evolutionary Biology 27:197-243
Conner JK (2002) Genetic mechanisms of floral trait correlations in a natural population. Nature 420 (6914):407-410. doi:10.1038/nature01105
Cresswell JE (1999) The influence of nectar and pollen availability on pollen transfer by individual flowers of oil-seed rape (Brassica napus) when pollinated by bumblebees (Bombus lapidarius). Journal of Ecology 87 (4):670-677. doi:10.1046/j.1365-2745.1999.00385.x
Dobson HEM, Danielson EM, Van Wesep ID (1999) Pollen odor chemicals as modulators of bumble bee foraging on Rosa rugosa Thunb. (Rosaceae). Plant Species Biology 14 (2):153-166. doi:10.1046/j.1442-1984.1999.00020.x
Galen C, Plowright RC (1985) The effects of nectar level and flower development on pollen carry-over in inflorescences of fireweed (*Epilobium angustifolium*) (Onagraceae) Canadian Journal of Botany-Revue Canadienne De Botanique 63 (3):488-491
Gómez JM, Bosch J, Perfectti F, Fernandez JD, Abdelaziz M, Camacho JPM (2008) Association between floral traits and rewards in Erysimum mediohispanicum (Brassicaceae). Annals of Botany 101 (9):1413-1420. doi:10.1093/aob/mcn053
Grafen A (1990) Biological signals as hadicaps Journal of Theoretical Biology 144 (4):517-546. doi:10.1016/s0022-5193(05)80088-8
Grose J (2011) Modelling and the fall and rise of the handicap principle. Biology & Philosophy 26 (5):677-696. doi:10.1007/s10539-011-9275-1
Harder LD, Barrett SCH (1992) The energy-cost of bee pollination for *Pontederia cordata* (Pontederiaceae) Functional Ecology 6 (2):226-233. doi:10.2307/2389759
Higham JP (2014) How does honest costly signaling work? Behavioral Ecology 25 (1):8-11. doi:10.1093/beheco/art097
Howell AD, Alarcon R (2007) Osmia bees (Hymenoptera : Megachilidae) can detect nectar-rewarding flowers using olfactory cues. Animal Behaviour 74:199-205. doi:10.1016/j.anbehav.2006.11.012
Huber FK, Kaiser R, Sauter W, Schiestl FP (2005) Floral scent emission and pollinator attraction in two species of Gymnadenia (Orchidaceae). Oecologia 142 (4):564-575. doi:10.1007/s00442-004-1750-9
Ida TY, Kudo G (2010) Modification of bumblebee behavior by floral color change and implications for pollen transfer in Weigela middendorffiana. Evolutionary Ecology 24 (4):671-684. doi:10.1007/s10682-009-9324-2
Johnstone RA (1997) The evolution o animal signals In: Krebs J, Davies N (eds) Behavioural ecology, an evolutionary approach, 4th edn Blackwell Scientific Publications, Oxford:155-178
Kadmon R, Shmida A (1992) Departure rules used by bees foraging for nectar - a field-test Evolutionary Ecology 6 (2):142-151. doi:10.1007/bf02270708
Knauer AC, Schiestl FP (2015) Bees use honest floral signals as indicators of reward when visiting flowers. Ecology Letters 18 (2):135-143. doi:10.1111/ele.12386
Knauer AC, Schiestl FP (2017) The effect of pollinators and herbivores on selection for floral signals: a case study in Brassica rapa. Evolutionary Ecology 31 (2):285-304. doi:10.1007/s10682-016-9878-8
Kotiaho JS (2001) Costs of sexual traits: a mismatch between theoretical considerations and empirical evidence. Biological Reviews 76 (3):365-376
Makino TT, Sakai S (2007) Experience changes pollinator responses to floral display size: from size-based to reward-based foraging. Functional Ecology 21 (5):854-863. doi:10.1111/j.1365-2435.2007.01293.x
Mitchell RJ (1993) Adaptive significance of Ipomopsis aggregata nectar production - Observation and experiment in the field Evolution 47 (1):25-35. doi:10.2307/2410115
Møller AP, Delope F (1994) Differential costs of a secondary sexual character - an experimental test of the handicap principle Evolution 48 (5):1676-1683. doi:10.2307/2410256
Odowd DJ (1980) Pearl bodies of a neotropical tree, *Ochroma pyramidale* - ecological implications American Journal of Botany 67 (4):543-549. doi:10.2307/2442294
Pélabon C, Thoene P, Hansen TF, Armbruster WS (2012) Signal honesty and cost of pollinator rewards in Dalechampia scandens (Euphorbiaceae). Annals of Botany 109 (7):1331-1339. doi:10.1093/aob/mcs091
Petanidou T, Goethals V, Smets E (1999) The effect of nutrient and water availability on nectar secretion and nectary structure of the dominant Labiatae species of Phrygana. Systematics and Geography of Plants 68 (1/2):233-244
Polnaszek TJ, Stephens DW (2014) Why not lie? Costly signals enforce honesty in an experimental signaling game. Integrative and Comparative Biology 54:E167-E167

Rader R, Howlett BG, Cunningham SA, Westcott DA, Newstrom-Lloyd LE, Walker MK, Teulon DAJ, Edwards W (2009) Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. Journal of Applied Ecology 46 (5):1080-1087. doi:10.1111/j.1365-2664.2009.01700.x

Raguso RA (2004) Why are some floral nectars scented? Ecology 85 (6):1486-1494. doi:10.1890/03-0410

Real LA, Rathcke BJ (1991) Individual variation in nectar production and its effect on fitness in Kalmia latifolia Ecology 72 (1):149-155. doi:10.2307/1938910

Rutter MT, Rausher MD (2004) Natural selection on extrafloral nectar production in Chamaecrista fasciculata: The costs and benefits of a mutualism trait. Evolution 58 (12):2657-2668. doi:10.1554/04-009

Sahli HF, Conner JK (2007) Visitation, effectiveness, and efficiency of 15 genera of visitors to wild radish, Raphanus raphanismum (Brassicaceae). American Journal of Botany 94 (2):203-209. doi:10.3732/ajb.94.2.203

Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28 (5):307-315. doi:10.1016/j.tree.2013.01.019

Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. New Phytologist 203 (1):257-266

Southwick EE (1984) Photosynthate allocation to floral nectar - a neglected energy investment Ecology 65 (6):1775-1779. doi:10.2307/1937773

Stanton M, Young HJ (1994) Selecting for floral character associations in wild radish, Raphanus sativus L. Journal of Evolutionary Biology 7 (3):271-285. doi:10.1046/j.1420-9101.1994.7030271.x

Stanton ML, Roy BA, Thiede DA (2000) Evolution in stressful environments. I. Phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. Evolution 54 (1):93-111

Szamado S (2011) The cost of honesty and the fallacy of the handicap principle. Animal Behaviour 81 (1):3-10. doi:10.1016/j.anbehav.2010.08.022

Tholl D, Boland W, Hansel A, Loreto F, Rose USR, Schnitzler JP (2006) Practical approaches to plant volatile analysis. Plant J 45 (4):540-560. doi:10.1111/j.1365-313X.2005.02612.x

Watanabe M, Ito A, Takada Y, Ninomiya C, Kakizaki T, Takahata Y, Hatakeyama K, Hinata K, Suzuki G, Takasaki T, Satta Y, Shiba H, Takayama S, Isogai A (2000) Highly divergent sequences of the pollen self-incompatibility (S) gene in class-I S haplotypes of Brassica campestris (syn. rapa) L. Febs Lett 473 (2):139-144. doi:10.1016/s0014-5793(00)01514-3

CHAPTER III

The effect of pollinators and herbivores on selection for floral signals: A case study in *Brassica rapa*

Knauer, A.C. and Schiestl, F.P.

Department of Systematic and Evolutionary Botany, Zollikerstrasse 107, University of Zurich, 8008 Zurich, Switzerland

ABSTRACT

Through their preferences for floral cues, pollinators, but also herbivores, can mediate selection on a variety of plant traits. Selection by mutualists and antagonists may not be independent from each other, however, as the selection on a trait through one interaction can depend on the presence or intensity of another interaction. Bumble bees (*Bombus terrestris*) and cabbage butterflies (*Pieris brassicae*) are both pollinators of *Brassica rapa*, but cabbage butterflies also use *B. rapa* as a host plant for their caterpillars. In a cage experiment we exposed *Brassica rapa* plants to a) bumble bees, b) cabbage butterflies, and c) bumble bees and cabbage butterflies together and analysed the resulting patterns of selection. We found an association between flower visitation and corolla size as well as the scent compound phenylacetaldehyde for both bumble bees and cabbage butterflies. Additionally, oviposition by butterflies was associated with the same two floral signals. Whereas corolla size was under positive selection in all three pollinator treatments, selection on phenylacetaldehyde was found only in the “pure” bumble bee treatment. Additionally, in plants exposed to both insect species negative correlational selection on corolla size and phenylacetaldehyde was found, as well as nonadditive selection on phenylacetaldehyde. These results demonstrate a strong overlap in the preferences for floral traits in pollinators and herbivores causing a conflict between the attraction of mutualists and the avoidance of antagonists. Further, our study shows that interactive effects on fitness by mutualists and antagonists can contribute to complex selection patterns.

INTRODUCTION

Most plant species interact with various mutualists and antagonists that shape the evolution of plant traits (e.g. Schemske and Bradshaw 1999; Gomez et al. 2015; Cornell and Hawkins 2003; Rosas-Guerrero et al. 2014). Because pollinators have a direct impact on plant fitness, they can mediate strong selection on plant traits by means of their preferences, morphology and behavior (Sletvold and Agren 2010; Sahli and Conner 2011). Floral traits that increase the attractiveness of flowers to mutualists are normally under positive directional selection (Galen 1989; Gomez 2003; Medel et al. 2003; Sahli and Conner 2011). Herbivores on the other hand can have direct negative impacts on plant fitness when feeding on flowers or fruits, but also indirectly impact fitness by reducing the resources available for the production of attractive flowers or seeds (Kessler and Halitschke 2009; Schiestl et al. 2014; McCall and Irwin 2006). Although herbivores can thus mediate various selective pressures on both floral and vegetative traits, plant traits attracting herbivores are normally found to be under directional negative selection (McCall et al. 2013; Strauss and Whittall 2006). Selection, however, is often not only shaped by pairwise interactions but also by the community of interacting organisms (Gomez 2005; Gomez 2008; Gomez 2003; Rey et al. 2006). This phenomenon is called “diffuse selection”, where selection on a trait through one interaction can depend on the presence or intensity of another interaction (Strauss and Irwin 2004).

Conflicting selection by mutualists and antagonists is one example of diffuse selection (Strauss and Irwin 2004). Many floral signals like color and scent have evolved to attract mutualists, mostly pollinators (Schiestl and Johnson 2013). However, antagonists can eavesdrop on such signals and use them to their own advantage. Especially for florivores and predispersal seed predators floral signals may not only be useful to find host plants but also give information about the quality

and quantity of food. Such eavesdropping can cause a trade off for the plant between the attraction of mutualists and the avoidance of antagonists (Theis and Adler 2012; Schiestl et al. 2011; Brody and Mitchell 1997) as well as opposite selection pressures on the same trait imposed by mutualists and antagonists (Gomez 2003; Gomez 2008). However, even when pollinators and herbivores mediate selection on different traits, these selective pressures can still influence each other. When traits are correlated, selection pressures on these traits are not independent (Strauss and Irwin 2004). Also, some combinations of traits can be favoured at the expense of other possible combinations, a phenomenon that is called correlational selection. In plant-animal interactions, combinations of floral signals causing high mutualist but low antagonist attraction at the same time should be under correlational selection (Herrera et al. 2002; Gomez 2008).

Finally, the presence of one species can affect the selection imposed by another species on plant traits, a phenomenon called nonadditive selection (Strauss and Irwin 2004). Different mechanisms could cause such an interaction. Firstly, the two species interacting with the plant may also interact with each other directly. Such direct interactions could cause a change in behavior and affect the selection imposed on plant traits. For example, some pollinators can deter others from flowers (Roubik 1978; Thomson 2004) which may cause changes in preferences for floral traits in the deterred species. Secondly, the presence of one species may affect the fitness effect of another one and thus the strength of selection it imposes on traits. For example, the degree of pollen limitation can influence the effect of herbivores on plant fitness (Herrera et al. 2002; Gomez 2005). Thirdly, the direction of the selection a species mediates on plant traits may be context dependent. Potentially this could be the case in pollinating herbivores: Pollinating herbivores might be beneficial when other pollinators are absent, but harmful when more efficient pollinators are present

(Reynolds et al. 2012; Thompson and Cunningham 2002). By their preference they may thus mediate opposite selection pressures on plant traits in different environments.

Although several studies investigated the interactive effect of pollinators and herbivores on plant fitness and selection (Wise and Hebert 2010; Galen and Cuba 2001; Gomez 2003; Gomez 2008; Gomez 2005; Rey et al. 2006; Parachnowitsch and Caruso 2008; Cariveau et al. 2004; Sletvold et al. 2015), we still lack deeper understanding of how conflicting, correlational and non-additive selection contribute to diffuse selection. This study aims to investigate selection on floral traits involved in the interactions of pollinators and herbivores with flowering *Brassica rapa* (Brassicaceae) plants. *B. rapa* flowers are visited by a variety of pollinators, including various species of bees and butterflies of the genus *Pieris* (Rader et al. 2009). However, *Pieris* butterflies not only pollinate flowers, but their caterpillars are specialist feeders on the plants and can have a strong negative effect on plant fitness (Smallegange et al. 2007). We used a cage experiment to expose *B. rapa* plants to either only bumble bees (*Bombus terrestris*), only cabbage butterflies (*Pieris brassicae*) or both insect species simultaneously. With this experiment we addressed the following questions: 1) Do bumble bees and cabbage butterflies overlap in their preferences for floral traits? 2) How does the presence of the pollinating herbivore *P. brassicae* change selection mediated by the bumble bee *B. terrestris*? 3) Is there correlational selection on trait combinations? 4) Do bumble bees and cabbage butterflies mediate nonadditive selection on floral traits?

MATERIALS AND METHODS

Study organism

B. rapa is a self-incompatible, annual or biennial herb native to Eurasia (Watanabe et al. 2000). It has a generalized pollination system with a wide variety of pollinators from the orders Hymenoptera, Diptera, Lepidoptera and Coleoptera (Rader et al. 2009). Whereas bee species are among the most important pollinators in terms of visitation rate and pollination efficiency (Sahli and Conner 2007; Rader et al. 2009), butterflies from the genus *Pieris* have a similarly high efficiency, but show low visitation rates (Sahli and Conner 2007; Rader et al. 2009). Further, their caterpillars are specialized to feed on Brassicaceae and adapted to overcome the glucosinolate defense system that is typical for this plant family (Smallegange et al. 2007; Wittstock et al. 2004). Although females lay their eggs on leaves, soon after hatching caterpillars prefer feeding on flowers, which can cause considerable losses in plant fitness (Smallegange et al. 2007). *Pieris* butterflies are thus both, pollinators and herbivores.

B. rapa seeds were collected in a natural population from about 100 individuals (population size over 1000 individuals, Maarssen, the Netherlands) and grown under standardized light, soil and watering conditions in a greenhouse. All plants were treated every second week with the pesticides Kendo and Thiovit (Maag, Dielsdorf, Switzerland) until start of flowering. The *B. terrestris* colony was purchased from Andermatt Biocontrol (Andermatt, Switzerland) and the hive was kept in a flight cage (3 x 1 x 1 m). The same bee hive was used during the whole experiment. Bumble bees were fed on pollen (purchased directly from beekeepers) and sugar solution (Apiinvert, Südzucker AG, Ochsenfurt). Additionally we exposed the bees to 20 to 30 flowering *B. rapa* plants for at least 3 hours before experimental use. *P. brassicae* butterflies and caterpillars were obtained from an in-house rearing which

was originally established by crossing three strains (see Bauer et al. 1998 for details). In 2015 another 80 caterpillars from 5 plants (in cabbage fields in the Region of Schaffhausen) were collected and crossed with the existing breeding line. For the rearing about 80 adults were kept in a flight cage (60 x 60 x 60 cm) and fed on sugar solution. A *B. rapa* plant was placed inside the cage for oviposition and replaced twice a week. From each plant about 30 hatched caterpillars were selected randomly and fed on cabbage leaves until pupation.

Plot experiments with pollinators

Plots of 36 *B. rapa* plants (6 x 6, 40 cm distance between plants) were set up in an outdoor cage (3 x 3 x 2 m) and exposed to pollinators according to the following three pollinator treatments: *a) pollinators only (12 bumble bees)*, *b) pollinating herbivores only (24 cabbage butterflies)* and *c) pollinators and pollinating herbivores (8 bumble bees and 16 cabbage butterflies)*. These numbers were chosen to obtain similar pollination services by both insect species. Further, the abundances of pollinators were limited to ensure pollen limitation. Although pollen limitation is not always the case (e.g. Parachnowitsch and Caruso 2008), it is a frequent condition in natural populations (Knight et al. 2005; Gomez et al. 2009). To reach appropriate oviposition rates by cabbage butterflies during exposure time we used a gender ratio of 3 females : 1 male which should not affect pollination as both sexes visit flowers for reward. Accordingly, infestation rates with *P. brassicae* caterpillars were within the rates typically found in nature (Ali and Rizvi 2007; Atalay and Hincal 1992). For each treatment three replicates were conducted leading to a total sample size of 108 plants per treatment. The replicates from different treatments were conducted alternately in the order as listed above to control for temporal effects. Each plot was kept in the cage for two subsequent sunny days. An additional replicate of treatment

c) was conducted at the beginning of the experiment. Because cool temperature slowed down the activity of cabbage butterflies at the time, this replicate was exposed for five days instead of two however. Therefore this data was only used in the analysis of insect behavior but not in the selection analysis. Cabbage butterflies were kept in the cage during the whole exposure time, whereas bumble bees were released to visit plants twice a day, each time for 15 min. All bees were marked with a dot after experimental use to avoid multiple usage of individuals (pseudoreplication). Plots were observed during four hours (two hours per day) and all pollinator visits to plants during this time were recorded. These observation times covered all bumble bee visits and the main activity period of cabbage butterflies.

At the end of the pollinator exposure all plants were scanned for the presence of *P. brassicae* eggs on the leaves. Eggs were left on the leaves for about 24 hours and then replaced with the same number of *P. brassicae* caterpillars in the second instar. This was done because *P. brassicae* caterpillars preferentially feed on flowers and buds, but not on fruits. Thus by waiting for a minimum of 3 days that eggs need to hatch, the flowers that had been exposed to pollinators would have been withered already and caterpillars would not have had a fitness effect (in nature however, *Pieris* caterpillars normally have a strong negative fitness effect as *B. rapa* flowers sequentially for several weeks). Caterpillars were placed on leaves but allowed to move freely on the plant. We restricted them to one plant individual by placing plants in plastic boxes (36 x 22 x 22 cm, open on the top), additionally we controlled infestation of all infested plants once a day and returned escaped caterpillars to the same plant. Caterpillars were removed from the plants after 7 days when they started to pupate. For each replicate with cabbage butterflies about 100 *P. brassicae* eggs were provided by the rearing to obtain caterpillars and butterflies for experimental use.

The controlled experimental design of this study is associated with a simplification of the pollinator guild as well as herbivore community compared to wild populations (we used only bumble bees and cabbage butterflies, whereas up to 31 species of flower visitors were documented in wild in *B. rapa* populations (Rader et al. 2009); for herbivore diversity in the wild see e.g. Root (1973)). Also, the abundances of pollinators as well as the composition of pollinator guilds and the presence of herbivores are highly variable in nature, both spatially and temporally (Root 1973; Galen 1989; Gross et al. 2016). Thus, selective patterns in wild populations can be expected to be more complex and variable over time and space than in a controlled experiment as ours. However, despite this limitation, there are also several advantages to such an experimental approach. First, environmental confounding factors, as for example resource availability, can be excluded. Second, all interactions can be recorded and thus selection can be directly traced to the organisms used. This can lead to a better understanding of the mechanisms causing selection patterns.

Measurement of floral and vegetative traits

Floral traits were measured for all plants used in the plot experiment. Further we measured floral and vegetative traits in an additional 25 plants to test for correlations between floral and vegetative traits. This was done to make sure the oviposition-preference for floral traits in cabbage butterflies was not caused by a correlation with any vegetative trait.

Floral traits

Volatiles were collected from inflorescences with the push-pull headspace collection method (Tholl et al. 2006). Inflorescences were enclosed in glass cylinders with two

openings (dimensions: 5 cm diameter, 25 cm height; all glass cylinders were treated previously with sigmacoate (Sigma-Aldrich, Buchs, Switzerland)). The bottom of the cylinder was closed with a teflon plate with a central hole allowing for the insertion of the peduncle without injuring it. For volatile collection glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into one opening and attached to a vacuum pump (DC06/04/20F, Fürgut GmbH, D-88459 Tannheim) with a silicon tube. Air was pulled through the Tenax tubes at a flow rate of 150 ml min^{-1}. After passing the tube, the air was circulated back (with the same flow rate) to the glass cylinder through another Tenax tube (Tenax GR 60/80, Scientific Instrument Services, Old York, NJ, USA), which was inserted through the other opening, to clean the incoming air. The number of flowers inside the cylinder was counted to calculate volatile amounts per flower. All collections took place between 12^{00} and 16^{00}hrs one day before pollinator exposure. After scent collection the Tenax tubes were stored at -30˚C until chemical analysis. For the analysis of floral volatiles, gas chromatography with mass selective detection (GC-MSD) was used. Samples were injected into a GC (Agilent 6890 N; Agilent Technologies, Santa Clara, CA, USA) using a Gerstel thermodesorption system (TDS3; Gerstel, Mühlheim, Germany) with cold injection (KAS4; Gerstel). The GC was equipped with a DB-5 column (0.32 mm ID, 0.25 lm film thickness, 30 m length), and helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Compound determination was done by comparing the spectra obtained from the natural samples with those of synthetic standard compounds. Standard compounds were also used for compound quantification using dose response curves for each volatile (Schiestl et al. 2014). A list of all analyzed compounds is given in the Supporting Information (Table S1).

Petal length and width were measured in three fully opened flowers per individual one day before pollinator exposure. Means of petal length and width were used to estimate the corolla size per flower as *π*length*width.*

Flower number was counted after pollinator exposure. Because flowers are open for approximately two days in *B. rapa*, we marked the lowest buds in all inflorescences two days before pollinator exposure and then counted the pedicles from markings to buds.

As measurements of attractiveness and fitness we used number of pollinator visits to inflorescences and relative seed set. To complete fruit development, plants were continuously watered for at least another three weeks after pollinator exposure. Afterwards, the number of seeds per individual was counted. Number of seeds represents an accurate estimate of the lifetime female fitness of the individuals since this species is monocarpic and reproduces only once during its life. Relative fitness was estimated by dividing the seed set of each individual by the population mean; this calculation was done separately for each replicate.

Vegetative traits

Vegetative traits were measured on the same day as floral traits. We measured plant height, the number of leaves and the amounts of 9 glucosinolate compounds. For glucosinolate analysis samples of fresh leaves (50-60 mg fresh weight from one young leaf per individual) were collected from each plant and immediately flash-frozen in liquid nitrogen. Samples were weighed and ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was collected in an Eppendorf tube, and 1 ml of ice-cold MeOH: water (70 : 30) with sinalbin (5 µg ml^{-1}) as internal standard was added. Samples were vortexed for 5 s and immediately incubated at 85°C for 10 min in a block heater which was simultaneously shaking with 600 rpm

(Eppendorf Thermomixer® comfort; Eppendorf, Hamburg, Germany). Subsequently, samples were placed in an ultrasonic bath for 10 min for further extraction (Typ AL 04-04; Advantage-Lab,Darmstadt, Germany). Finally, samples were centrifuged at 14000 g for 10 min (Sorvall RMC 14, Kendro Laboratory Products, Asheville, NC, USA) and the supernatant was transferred to a new tube and stored at -20°C until ultrahighpressureliquid chromatography (UHPLC) analysis. Identification and quantification of glucosinolates in the extracts was done by UHPLC/MS as described in Schiestl et al. (2014).

Statistical analysis

Before statistical analysis, the number of seeds was ln(1+x) transformed and all volatile variables BoxCox-transformed to obtain homogeneity of variance, and approach normal distributions. Floral volatiles emitted by less than 90% of the individuals were excluded from the dataset to reduce the number of explanatory variables. Variable standardization of floral traits by z-transformation was done within replicates to eliminate differences in means and variance. All statistical analysis was performed with R 3.2.3 (the R project for statistical computing).

Attraction of bumble bees and cabbage butterflies in B. rapa

The association between pollinator and herbivore attraction was calculated using generalized mixed effect models with poisson distribution. The number of visits by either bumble bees or cabbage butterflies was fitted as response and the presence of eggs (present vs. absent) as explanatory variables; the replicate was included as random effect. This model was calculated with the data from pollinator treatment c) (bumble bees and cabbage butterflies).

Further, we used generalized mixed models to test for the attractiveness of traits for bumble bees and cabbage butterflies. We fitted a model with number of visits of one insect species as response and the standardized floral traits as explanatory variables using a poisson distribution. To test for a preference in oviposition by cabbage butterfly females we fitted a model with presence of eggs (present vs. absent) as response and the standardized floral traits as explanatory variables using a binomial distribution. The pollinator treatment (see above under plot experiments with pollinators) was included as random effect. We used presence of eggs instead of number of eggs as response variable because the number of eggs a female lays depends on her age, size and the temperature in addition to the preference for the plant (Gossard and Jones 1977; Jones et al. 1982). Additionally, we used generalized linear models to test for an effect of the presence of one pollinator species on the preferences of the other. Models were fitted as described above with the only difference that the pollinator treatment was now fitted as a fixed effect interacting with the floral traits. A significant interaction would indicate the alteration of a preference in the presence of the other pollinator species.

Fitness effects of flower visitation and herbivory

A one-way ANOVA was used to compare the number of seeds of non-infested plants between pollinator treatments. To test for the effects of flower visitation and herbivory on plant fitness we used a mixed effect model fitting the number of seeds as response and the infestation with caterpillars (infested vs. non-infested) and the number of visits by bumble bees and cabbage butterflies as explanatory variables. To test for nonadditive effects by pollinators and herbivores the interaction between infestation and pollinator visits was included. The replicate (see above under plot experiments with pollinators) was included as random effect. This model was

calculated with the data from pollinator treatment c) (bumble bees and cabbage butterflies).

Directional selection

To test for directional selection on floral traits we fitted a multivariate model for each pollinator treatment separately (Lande and Arnold 1983). The relative fitness (seed set/(mean seed set of replicate)) was included as response in the model whereas the standardized floral traits were included as explanatory variables. Because the number of seeds was bimodally distributed due to herbivory in the pollinator treatments b) and c), we included infestation with caterpillars as random effect in these treatments (when infestation was included as fixed effect, no significant interaction with any floral trait was detected) whereas no random effect was included in pollinator treatment a). By this approach the model's requirements (normal distribution of residuals) can be fulfilled. Additionally the analysis is more independent from the degree of herbivory, which indeed was high in our experiment due to the lack of predation on caterpillars. To test for differences in selection gradients between pollinator treatments we conducted an ANCOVA.

Correlational selection

Because bumble bees and/or cabbage butterflies showed a preference for flower number, corolla size and the amounts of phenylacetaldehyde and (E)-α-farnesene, we tested for correlational selection on these traits. The same model as to measure directional selection was used but we additionally included all pairwise interactions between the floral traits as explanatory variables into the model (the result of this analysis did not change when quadratic terms were included additionally). A significant interaction would indicate correlational selection on a specific trait

combination. To test for differences in correlational selection between pollinator treatments we conducted an ANCOVA.

Nonadditive selection

To test for nonadditive selection by bumble bees and cabbage butterflies we compared the selection gradients in plants that had been exposed to bumble bees and cabbage butterflies together (pollinator treatment c) with the gradients expected under additive selection. Under additive selection, when both interacting species are present, the selection gradient on a plant trait is the average of the gradients imposed by each species when present alone (Sahli and Conner 2011). Thus, the expected gradients were calculated as *(1/2 * selection gradient in pure bumble bee treatment) + (1/2 * selection gradient in pure cabbage butterfly treatment).* This formula is valid for both, positive selection imposed by pollination as well as negative selection mediated by herbivory. For pollination, both "pure" treatments have very similar average fitness in non-infested plants (123 ± 13 and 154 ± 15 (mean ± s.e. seedset)). This shows that both pollinator species have a similar overall pollination efficiency. As we used them in the mixed treatment in equal proportions (compared to pure treatments), they should contribute equally to pollination. Also, because we used relative fitness as response, the expected gradient can be calculated as the average of the pure gradients as long as both pollinators contribute equally to fitness (the fitness effect of each pollinator in the mixed treatment does not have to be half of the effect in the pure treatment). Therefore, with respect to pollination, the expected selection gradient can be calculated with the formula above. For herbivory, infestation rate in the mixed treatment was about 1/2 of the pure butterfly treatment (31 ± 3 % vs 52 ± 2 %), whereas the infestation in the pure bumble bee treatment was zero. Thus, also with respect to herbivory, the expected value can be calculated

with the formula above**.** Finally, we tested for non-additive selection using the analysis established by Sali and Conner (2011) (see also SI for a detailed description).

RESULTS

Attraction of bumble bees and cabbage butterflies to *B. rapa*

For individual bumble bees we observed a mean visitation rate of 28.1 plants * hour^{-1}, whereas for cabbage butterflies visitation rate was only 1.2 plants * hour^{-1}. Cabbage butterfly females oviposited on 0.50 plants day^{-1}, which led to an infestation of 52 ± 2% (mean ± s.e.) of plants in the treatment with cabbage butterfly exposure and to an infestation of 31 ± 3% in plants exposed to both insect species, both rates are inside the range found in natural cabbage fields (Atalay and Hincal 1992).

Pollinator attraction was positively associated with herbivore attraction in plants exposed to bumble bees and cabbage butterflies together. Plants that attracted cabbage butterfly females for oviposition also received higher number of visits to inflorescences by bumble bees (estimate ± s.e. = 0.41 ± 0.12, z = 3.319, P < 0.001) as well as by cabbage butterflies (0.26 ± 0.10, z = 2.683, P = 0.007) (Figure 1).

In bumble bees, flower number, corolla size and the amounts of phenylacetaldehyde and (*E*)-α-farnesene had a significant positive effect on the number of visits to inflorescences (Table 1, Figure 2). However, we also found a significant interactive effect of the pollinator treatment and the floral volatile p-anisaldehyde on flower visitation in bumble bees. Bees showed a significantly higher apparent preference for the floral volatile p-anisaldehyde when cabbage butterflies were present than when they were absent (β = 0.35 ± 0.18, z = 1.959, P = 0.050). In cabbage butterflies, number of flowers, corolla size and the amount of

phenylacetaldehyde had a significant positive effect on flower visitation, whereas the compound 1-butene-4-isothiocyanate had a significant negative effect (Table 1). In cabbage butterflies, the preferences for floral traits were not affected by the presence of bumble bees. Further, only corolla size and phenylacetaldehyde had a significant positive effect on oviposition (Table 1, Figure 3). Corolla size was not correlated with any vegetative trait, whereas the amount of phenylacetaldehyde was only significantly correlated with the concentration of the glucosinolate gluconapin in leaves; however, this correlation was negative and thus cannot explain the association between this scent compound and oviposition ($r = -0.52$, $P = 0.007$, Table S2).

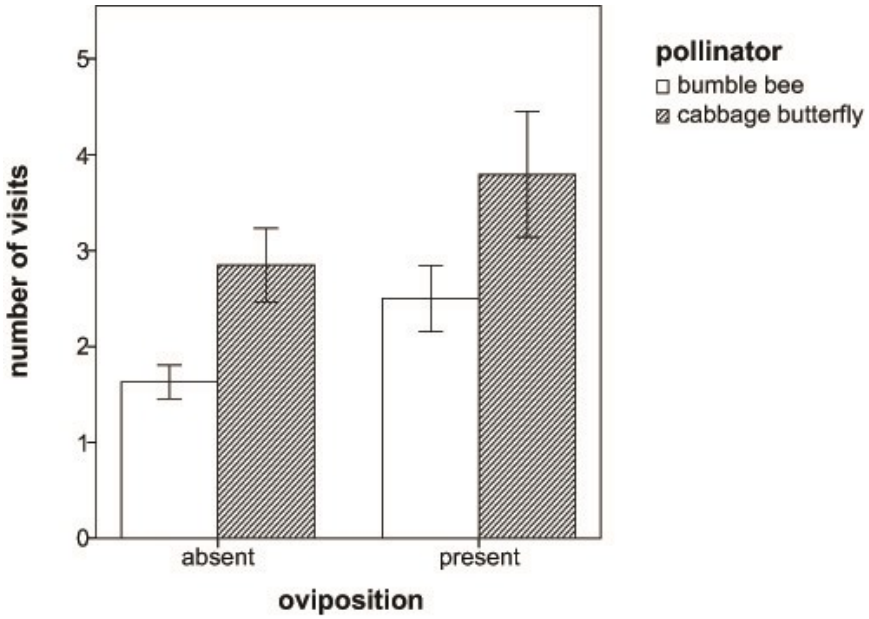


Figure 1 Association between herbivore and pollinator attraction. Plants that attracted cabbage butterfly females for oviposition also received significantly more visits to inflorescences by bumble bees (estimate ± s.e. = 0.41 ± 0.12, $z = 3.319$, $P < 0.001$) as well as by cabbage butterflies (0.26 ± 0.10, $z = 2.683$, $P = 0.007$).

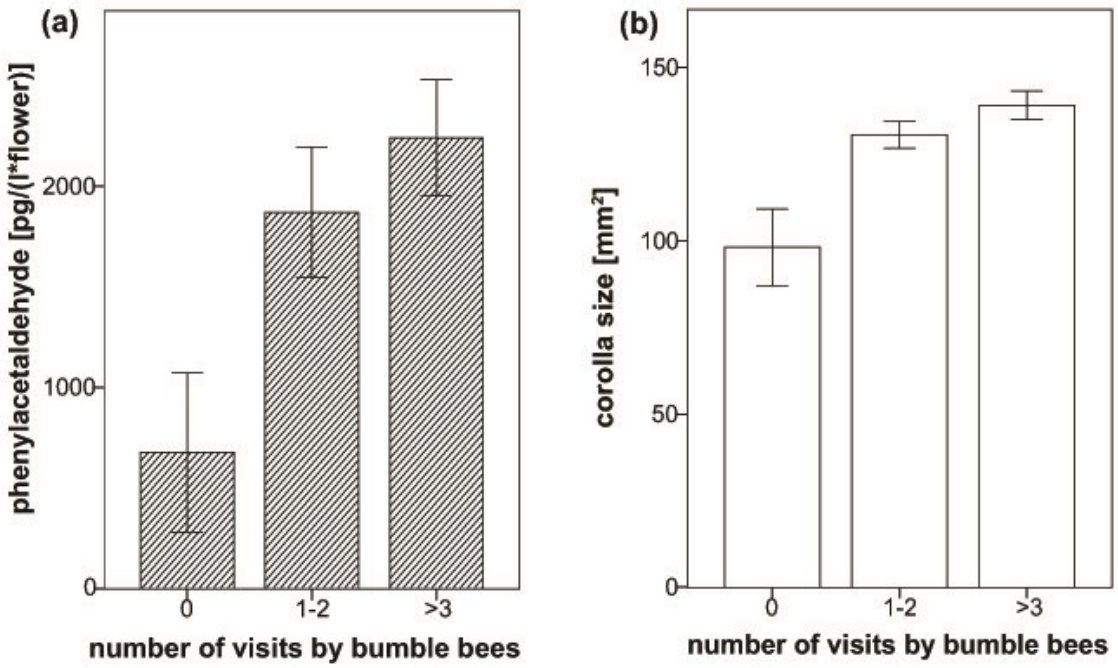


Figure 2 Bumble bees were attracted to *B. rapa* flowers by **a)** high amounts of the floral volatile phenylacetaldehyde (generalized linear model: β = 0.15 ± 0.06, z = 2.579, P = 0.010) and **b)** large corollas (generalized linear model: β = 0.24 ± 0.05, z = 4.586, P < 0.001). Bumble bee visits were only categorized for graphical display (to obtain similar sample sizes in bars), but not for statistical analysis.

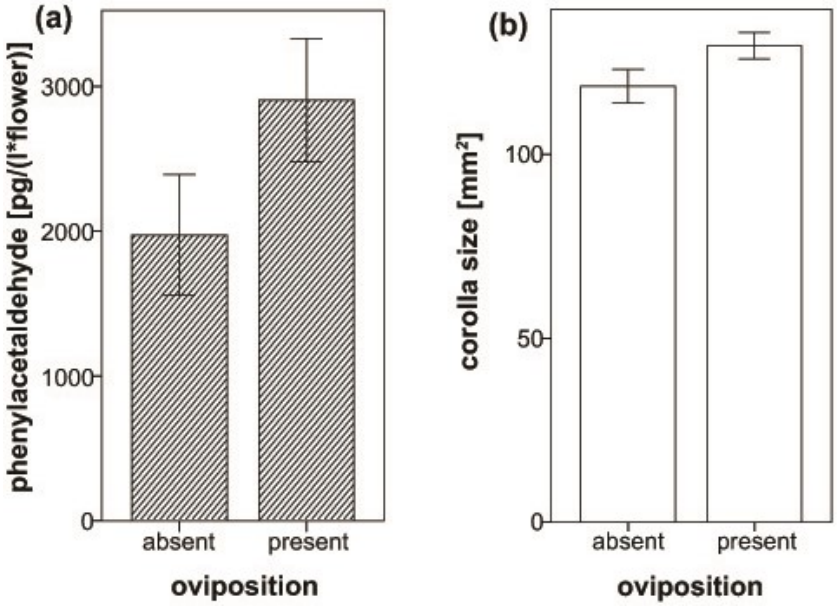


Figure 3 For oviposition, cabbage butterflies were attracted by **a)** high amounts of the floral volatile phenylacetaldehyde (generalized linear model: β = 0.41 ± 0.17, z = 2.408, P = 0.016) and **b)** large corollas (generalized linear model: β = 0.45 ± 0.16, z = 2.766, P = 0.006).

Fitness effects of flower visitation and herbivory

Plants exposed to bumble bees had a mean fruit set of 36 ± 26% (mean ± s.d.) and 123 ± 137 seeds per individual, plants exposed to cabbage butterflies had a mean fruit set of 23 ± 28% and produced 90 ± 134 seeds, whereas plants exposed to both insect species had a fruit set of 26 ± 28% and produced 86 ± 109 seeds per individual. The mean number of seeds in non-infested plants (from all three pollinator treatments) was 127 ± 135, but herbivory reduced the number of seeds to 33 ± 73 seeds on average. Number of seeds per individual did not differ significantly between pollinator treatments when only non-infested plants were considered (F_2 = 1.637, P = 0.20). While flower visitation by bumble bees and cabbage butterflies had a significant positive effect on plant fitness (bumble bees: estimate ± s.e. = 20.5 ± 4.8, t = 4.292, P < 0.01; cabbage butterflies: estimate ± s.e. = 6.8 ± 2.0, t = 3.305, P < 0.01), herbivory by caterpillars had a significant negative effect (estimate ± s.e. = -04.5 ± 19.0, t = -5.489, P < 0.001, see also Figure S1 for the association between plant fitness and the degree of infestation). Also, there was a significant interaction between the effect of herbivory and the effect of flower visitation by bumble bees. In herbivore infested plants the effect of bee visits on fruit set was significantly lower than in non-infested plants (estimate ± s.e. = -18.9 ± 9.5, t = -1.999, P = 0.046; Figure 4).

Selection on floral traits

In plants exposed to bumble bees, the number of flowers, corolla size and the floral volatiles phenylacetaldehyde and (*E*)-α-farnesene were under significant positive directional selection, while the floral volatile 1-butene-4-isothiocyanate was under significant negative selection. In plants exposed to cabbage butterflies, only corolla size was under significant positive selection. In plants exposed to both types of

pollinators, we found significant positive selection on the number of flowers, corolla size and (*E*)-α-farnesene, whereas methyl salicylate was under significant negative selection (Table 2, Figure 5, please also see Table S3 for correlations between floral traits). However, we could not detect a significant trait x pollinator treatment interaction in the ANCOVA.

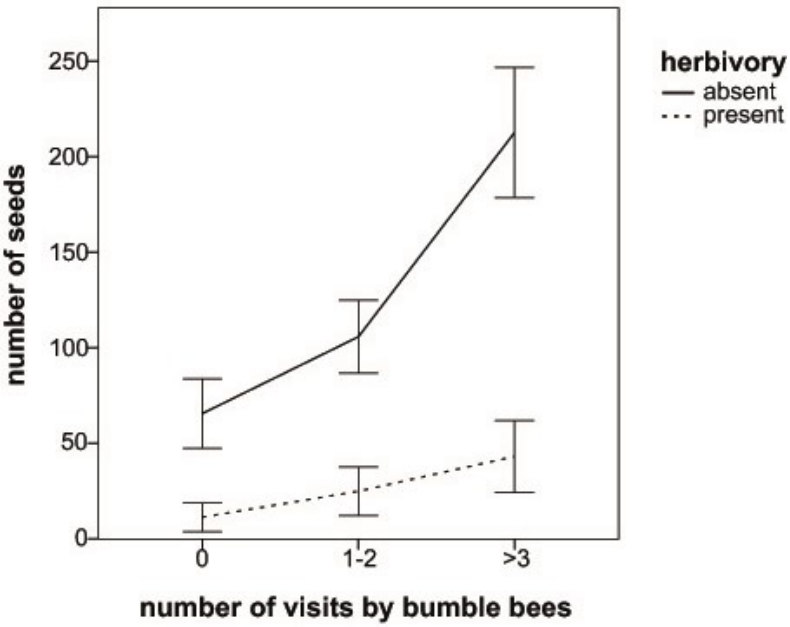


Figure 4 The effect of bumble bee visits on plant fitness depended on the presence of herbivory by cabbage butterflies (estimate ± s.e. = -18.9 ± 9.5, t = -1.999, P = 0.046). Bumble bee visits were only categorized for graphical display, but not for statistical analysis.

We only found correlational selection in the pollinator treatment combining bumble bee and cabbage butterfly presence. In this treatment, significant negative correlational selection on corolla size and phenylacetaldehyde was found (Table 3). These two traits are not correlated with each other in *B. rapa* (Table S3). However, we could not detect a significant effect of the pollinator treatment on correlational selection in the ANCOVA.

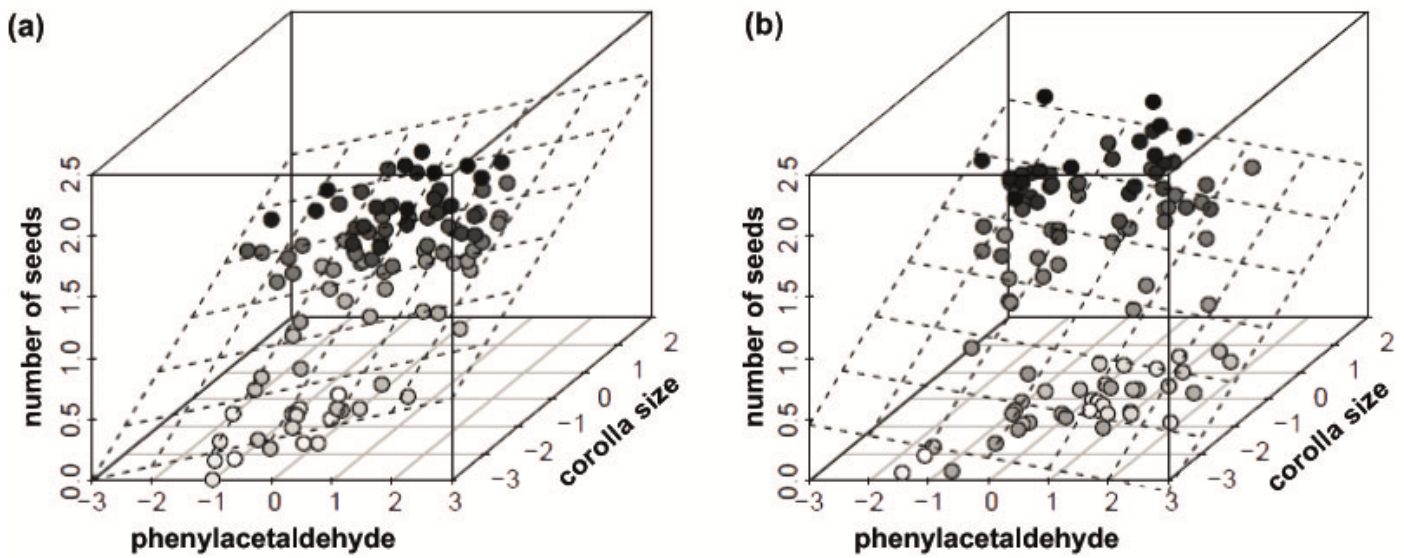


Figure 5 a) In plants exposed to bumble bees, corolla size and the floral volatile phenylacetalehyde were under significant positive selection (multivariate regression: corolla size: β = 0.22 ± 0.04, t = 4.966, P < 0.001; phenylacetaldehyde: β = 0.15 ± 0.5, t = 3.274, P = 0.001). **b)** In plants exposed to bumble bees and cabbage butterflies together, we only found significant selection on corolla size (β = 0.22 ± 0.08, t = 2.796, P = 0.005), but not on phenylacetaldehyde. Additionally, significant negative correlational selection on these two traits was found in this treatment (β = -0.17 ± 0.08, t = -2.149, P = 0.029).The color gradient reflects the sorted numbers of seeds (from white to black for small values to big values) for better visualization.

Table 1 Attraction of bumble bees and cabbage butterflies by floral traits. To test for the attractiveness of floral traits to pollinators the number of visits to inflorescences was used as the response variable, to test for the attractiveness of floral traits to cabbage butterflies when selecting plants for oviposition the oviposition (eggs present or absent) was fitted as response.

	Bumble bee			**Cabbage butterfly**					
	Visits			Visits			Oviposition		
Floral trait	β ± s.e.	z	P	β ± s.e.	z	P	β ± s.e.	z	P
Flower number	0.34 ± 0.04	7.659	**< 0.001**	0.21 ± 0.03	6.382	**< 0.001**	0.04 ± 0.14	0.303	0.76
Corolla size	0.24 ± 0.05	4.586	**< 0.001**	0.15 ± 0.04	3.945	**< 0.001**	0.45 ± 0.16	2.766	**0.006**
Phenylacetaldehyde	0.15 ± 0.06	2.579	**0.010**	0.09 ± 0.04	2.187	**0.029**	0.41 ± 0.17	2.408	**0.016**
Benzaldehyde	-0.10 ± 0.08	-1.211	0.23	-0.04 ± 0.06	-0.668	0.50	0.11 ± 0.24	0.466	0.64
1-Butene-4- isothiocyanate	-0.01 ± 0.05	-0.061	0.95	-0.11 ± 0.04	-2.812	**0.005**	-0.04 ± 0.16	-0.258	0.62
Methyl benzoate	0.03 ± 0.07	0.476	0.63	0.04 ± 0.05	1.902	0.37	0.01 ± 0.20	-0.015	0.96
Methyl salicilate	-0.01 ± 0.06	-0.224	0.82	0.03 ± 0.04	0.745	0.46	0.22 ± 0.17	1.281	0.20
p-Anisaldehyde	0.03 ± 0.09	0.288	0.77	-0.02 ± 0.06	-0.299	0.76	-0.17 ± 0.23	-0.718	0.47
E-α-Farnesene	0.18 ± 0.05	3.522	**< 0.001**	-0.05 ± 0.04	-1.304	0.19	-0.16 ± 0.16	-0.943	0.35

Table 3 Correlational selection gradients on floral traits attracting pollinators and/or herbivores in the three pollinator treatments.

	Bumble bee			**Bumble bee & Cabbage butterfly**			**Cabbage butterfly**		
Floral trait	β ± s.e.	t	P	β ± s.e.	t	P	β ± s.e.	t	P
Flower number x corolla size	0.01 ± 0.05	0.263	0.79	0.02 ± 0.08	0.261	0.79	-0.09 ± 0.08	-1.099	0.27
Flower number x phenylacetaldehyde	-0.03 ± 0.05	-0.608	0.54	0.10 ± 0.08	1.274	0.20	-0.10 ± 0.11	-0.876	0.38
Flower number x (E)-α-farnesene	0.03 ± 0.04	0.808	0.42	-0.02 ± 0.07	-0.274	0.78	-0.07 ± 0.10	-0.651	0.52
Corolla size x phenylacetaldehyde	-0.09 ± 0.05	-1.859	0.07	-0.17 ± 0.08	-2.186	**0.029**	-0.07 ± 0.10	-0.705	0.48
Corolla size x (E)-α-farnesene	-0.03 ± 0.05	-0.556	0.58	0.03 ± 0.07	0.399	0.69	0.06 ± 0.10	0.660	0.51
Phenylacetaldehyde x (E)-α-farnesene	0.01 ± 0.05	0.351	0.73	-0.01 ± 0.08	-0.131	0.90	-0.10 ± 0.08	-1.202	0.23

Table 2 Directional selection gradients on floral traits in the three pollinator treatments using relative seed set as fitness estimate.

	Bumble bee			**Bumble bee & Cabbage butterfly**			**Cabbage Butterfly**		
Floral trait	β ± s.e.	t	P	β ± s.e.	t	P	β ± s.e.	t	P
Flower number	0.13 ± 0.04	2.999	**0.003**	0.23 ± 0.08	2.948	**0.003**	0.07 ± 0.09	0.766	0.44
Corolla size	0.22 ± 0.04	4.945	**< 0.001**	0.22 ± 0.08	2.796	**0.005**	0.26 ± 0.09	2.695	**0.007**
Benzaldehyde	-0.03 ± 0.08	-0.453	0.65	-0.02 ± 0.12	0.170	0.86	0.17 ± 0.14	1.279	0.40
Phenylacetaldehyde	0.15 ± 0.05	2.798	**0.006**	-0.09 ± 0.08	-1.098	0.27	0.08 ± 0.10	0.834	0.18
1-Butene-4- isothiocyanate	-0.11 ± 0.04	-2.589	**0.011**	0.07 ± 0.08	0.825	0.41	-0.13 ± 0.09	-1.331	0.20
Methyl benzoate	0.11 ± 0.06	1.767	0.08	0.15 ± 0.10	1.581	0.11	0.03 ± 0.12	0.240	0.81
Methyl salicilate	-0.05 ± 0.05	-0.997	0.32	-0.17 ± 0.08	-2.149	**0.032**	-0.02 ± 0.10	-0.213	0.83
p-Anisaldehyde	0.01 ± 0.07	-0.165	0.87	0.09 ± 0.11	0.852	0.39	-0.13 ± 0.13	-1.038	0.30
E-α-Farnesene	0.11 ± 0.04	2.494	**0.014**	0.15 ± 0.07	2.028	**0.042**	0.06 ± 0.10	0.627	0.53

We found significant nonadditive selection on the floral volatile phenylacetaldehyde (t = 2.619, P = 0.009). The actual selection gradient was 0.20 ± 0.08 lower than expected under the assumption of additive selection. Additionally we found significant nonadditive selection on 1-butene-4-isothiocyanate (estimate = 0.19 ± 0.08, t = -2.363, P = 0.018).

DISCUSSION

Although many studies have measured selection imposed by pollinators and herbivores (Gomez 2008; Rey et al. 2006; Gomez 2003; Cariveau et al. 2004; Galen and Cuba 2001; Agren et al. 2013), many of them have failed to detect a conflict between the attraction of mutualists and antagonists (Kudoh and Whigham 1998; Cariveau et al. 2004; Parachnowitsch and Caruso 2008; Brody and Mitchell 1997; Sanchez-Lafuente 2007). Further, most of these studies measured only a small number of traits with a strong bias towards visual and morphological floral traits (but see Theis et al. 2014). This leaves a gap in our understanding of how strongly pollinators and herbivores overlap in their preferences and what the consequences are for the selection on floral traits. Our study demonstrates a strong overlap in the preferences of a frequent pollinator (bumble bee) and a specialist herbivore (cabbage butterfly) for the floral traits in *B. rapa*. Further, bumble bees and cabbage butterflies also influenced each other's selective pressures indirectly, namely by correlational and nonadditive selection. Overall, our study gives new insights into how pollinators, herbivores and the interactions between the two selective agents shape overall selection on floral traits.

Whereas pollinators are known to rely on floral signals to find floral rewards (Schiestl and Johnson 2013; Knauer and Schiestl 2015; Omura et al. 1999), oviposition preferences in herbivores are generally thought to be mediated by

vegetative traits, such as leaf glucosinolates for *Pieris* butterflies (Du et al. 1995; Stadler et al. 1995). However, in our experiment two floral traits, corolla size and phenylacetaldehyde, not only attracted bees and cabbage butterflies to flowers, but also cabbage butterflies to oviposit on leaves. Because none of the traits was positively correlated to leaf glucosinolate contents, it is unclear why cabbage butterflies might use them for oviposition choice. First, both traits have been shown to be correlated with nectar and pollen amount present in flowers (Knauer and Schiestl 2015). Such honest signals can be used by pollinators to efficiently find food sources (Knauer and Schiestl 2015) and thus may also allow for the identification of abundant resources for caterpillars. *Pieris brassicae* caterpillars are known to preferentially feed on flowers in *Brassica* and flower feeding sustains a higher growth rate than leaf feeding (Smallegange et al. 2007). Second, it has been shown that high amounts of scent decrease the attractiveness of *Pieris*-infested plants for the parasitoid *Cortesia glomerata*, a major specialist parasitoid in *Pieris* butterflies (Schiestl et al. 2014). Ovipositing on plants with high phenylacetaldehyde emission might thus provide protection from parasitoids for caterpillars.

A trade off between the attraction of pollinators and herbivores has been documented in several plant species (Cariveau et al. 2004; Theis 2006; Gomez 2008; Gomez 2003; Theis and Adler 2012; Galen and Cuba 2001) and can cause conflicting selection on the traits attracting mutualists and antagonists at the same time (Strauss and Irwin 2004). Consistently, in our study pollinator attraction was positively associated with herbivore attraction, which was probably caused by their overlap in preference for floral traits. Due to the positive fitness effect of pollinators but the negative effect of herbivores, this association causes conflicting selection on the respective floral traits. Whereas persistent directional or stabilizing selection can deplete genetic variation in traits, conflicting directional selection can enhance

variation (Siepielski and Benkman 2010). Fluctuating abundances of mutualists and antagonists over time and space should cause changes in the overall selective pressure on traits being under conflicting selection, maintaining the variance in these traits. In *B. rapa*, phenylacetaldehyde has a high heritability (Zu et al. 2016) and was the only scent compound we found to be under conflicting selection by bumble bees and cabbage butterflies. Indeed, phenylacetaldehyde has a variance 2.4 times higher than (*E*)-α-farnesene, the second scent compound under selection by bumble bees but not herbivores (see Table S4). Conflicting selection on phenylacetaldehyde thus might actually have contributed to its variability.

Private channels allow plants to exclusively communicate with only a subset of interacting animals due to a poor detection of the signal in unintended receivers (Raguso 2008). In our study, the floral volatile (*E*)-α-farnesene was associated with flower visitation by bumble bees, whereas cabbage butterflies did not show any preference for this compound, making it a possible private channel for bumble bee attraction. Indeed, (*E*)-α-farnesene can be perceived by *B. terrestris* antenna (EAD-active compound) and has been linked to the attraction of bees to flowers (Knauer and Schiestl 2015; Valterova et al. 2007; Blight et al. 1997). In *Pieris* butterflies on the other hand (*E*)-α-farnesene, unlike phenylacetaldehyde, was never shown to be perceivable or attractive (Honda et al. 1998; Omura et al. 1999). Unlike corolla size and phenylacetaldehyde, (*E*)-α-farnesene was not under conflicting and negative correlational selection in plants exposed to bumble bees and cabbage butterflies. Whereas the negative correlational selection on phenylacetaldehyde and corolla size makes the selection on these traits depend on each other, the selection on (*E*)-α-farnesene is independent from the selection on other floral traits. Also, Zu *et al.* (2016) showed that (*E*)-α-farnesene emission evolves rapidly and independently from corolla size and phenylacetaldehyde emission. All together, even under high

herbivore pressure, the emission of (*E*)-α-farnesene can be expected to be maintained over time and successfully attract pollinators. Such private channels may offer plants the possibility to successfully escape herbivore infestation but maintain the attraction of mutualists.

So far, little is known about the occurrence of nonadditive selection in plant-insect interactions and the mechanisms causing it (but see Sletvold et al. 2015; Sahli and Conner 2011; Juenger and Bergelson 1998). We document here nonadditive selection on the floral volatile phenyacetaldehyde, which attracts pollinators as well as pollinating herbivore to *B. rapa* flowers. Interestingly, neither bumble bees nor cabbage butterflies were affected in their preference for phenylacetaldehyde by each other's presence. A direct interaction between the two insect species thus did not contribute to the nonadditive selection. However, two other mechanisms may have contributed to the selection pattern; the first one is the nonadditive effect of pollination and herbivory on plant fitness. Flower visitation by bumble bees had a smaller effect on plant fitness when plants were infested with caterpillars than in uninfested plants. Such nonadditive effects between mutualists and antagonists are common and can occur when herbivores destroy previously pollinated flowers or fruits reducing the fitness effect of pollination (Gomez 2005; Herrera et al. 2002). The second mechanism is the context-dependence of the average fitness-effect of cabbage butterflies. In *B. rapa*, cabbage butterflies have to be classified as mutualists in the absence of other pollinators, because then their presence allows reproduction (*B. rapa* is self-incompatible). In the presence of more efficient pollinators, however, they are antagonists, as on average they reduce plant fitness by herbivory. Cabbage butterflies may thus mediate opposite selection pressures on attractive floral traits in the absence and presence of bumble bees, possibly contributing to the nonadditive selection.

Several studies have investigated the effect of pollinators and herbivores on the selection on floral traits in the field (Cariveau et al. 2004; e.g. Gomez 2003; Gomez 2008). Our study is the first one investigating this topic in a completely controlled experiment which gives important insights into the principles of conflicting, correlational and nonadditive selection. However, there are still gaps in our knowledge, for example about the frequency of conflicting selection and its contribution to the maintenance of trait variability, the relevance of private channels and correlational selection in trait evolution as well as the mechanisms behind nonadditive selection.

ACKNOWLEDGEMENT

We would like to thank Rayko Jonas and Markus Meierhofer for their help with plant cultivation. Also we would like to thank Tanja Christoffel for the insect rearing and Franz Huber for his support in the GC lab. The research leading to these results has received funding from the European Union's Seventh Framework Program ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement n° 281093.

REFERENCES

Agren J, Hellstrom F, Torang P, Ehrlen J (2013) Mutualists and antagonists drive among-population variation in selection and evolution of floral display in a perennial herb. Proceedings of the National Academy of Sciences of the United States of America 110 (45):18202-18207. doi:10.1073/pnas.1301421110

Ali A, Rizvi PQ (2007) Developmental response of cabbage butterfly, *Pieris brassicae L.* (Lepidoptera : Pieridae) on different cole crops under laboratory and field condition. Asian Journal of Plant Sciences 6 (8):1241-1245

Atalay R, Hincal P (1992) Investigations on the species of Pieridae (Lepidoptera) which are harmful to plants belonging to the family Cruciferae with their importance in Izmir and its vicinity and the biology of the large white butterfly (Pieris brassicae (L.)) along with the factors affecting its population fluctuations. Doga Turk Tarim ve Ormancilik Dergisi 16 (1):271-286

Bauer E, Trenczek T, Dorn S (1998) Instar-dependent hemocyte changes in Pieris brassicae after parasitization by Cotesia glomerata. Entomologia Experimentalis Et Applicata 88 (1):49-58. doi:10.1046/j.1570-7458.1998.00345.x

Blight MM, LeMetayer M, Delegue MHP, Pickett JA, MarionPoll F, Wadhams LJ (1997) Identification of floral volatiles involved in recognition of oilseed rape flowers, Brassica napus by honeybees,

Apis mellifera. Journal of Chemical Ecology 23 (7):1715-1727. doi:10.1023/B:JOEC.0000006446.21160.c1
Brody AK, Mitchell RJ (1997) Effects of experimental manipulation of inflorescence size on pollination and pre-dispersal seed predation in the hummingbird-pollinated plant Ipomopsis aggregata. Oecologia 110 (1):86-93. doi:10.1007/s004420050136
Cariveau D, Irwin RE, Brody AK, Garcia-Mayeya LS, von der Ohe A (2004) Direct and indirect effects of pollinators and seed predators to selection on plant and floral traits. Oikos 104 (1):15-26. doi:10.1111/j.0030-1299.2004.12641.x
Cornell HV, Hawkins BA (2003) Herbivore responses to plant secondary compounds: A test of phytochemical coevolution theory. American Naturalist 161 (4):507-522. doi:10.1086/368346
Du YJ, Vanloon JJA, Renwick JAA (1995) Contact chemoreception of oviposition-stimulating glucosinolates and an oviposition-deterrent cardenolide in 2 subspecies of *Pieris napi* Physiological Entomology 20 (2):164-174. doi:10.1111/j.1365-3032.1995.tb00813.x
Galen C (1989) Measuring pollinator-mediated selection on morphometric floral traits - Bumble bees and the alpine sky pilot, Polemonium viscosum Evolution 43 (4):882-890. doi:10.2307/2409315
Galen C, Cuba J (2001) Down the tube: Pollinators, predators, and the evolution of flower shape in the alpine skypilot, Polemonium viscosum. Evolution 55 (10):1963-1971
Gomez JM (2003) Herbivory reduces the strength of pollinator-mediated selection in the Mediterranean herb Erysimum mediohispanicum: Consequences for plant specialization. American Naturalist 162 (2):242-256
Gomez JM (2005) Non-additive effects of herbivores and pollinators on Erysimum mediohispanicum (Cruciferae) fitness. Oecologia 143 (3):412-418. doi:10.1007/s00442-004-1809-7
Gomez JM (2008) Sequential conflicting selection due to multispecific interactions triggers evolutionary trade-offs in a monocarpic herb. Evolution 62 (3):668-679. doi:10.1111/j.1558-5646.2007.00312.x
Gomez JM, Abdelaziz M, Camacho JPM, Munoz-Pajares AJ, Perfectti F (2009) Local adaptation and maladaptation to pollinators in a generalist geographic mosaic. Ecology Letters 12 (7):672-682. doi:10.1111/j.1461-0248.2009.01324.x
Gomez JM, Perfectti F, Lorite J (2015) The role of pollinators in floral diversification in a clade of generalist flowers. Evolution 69 (4):863-878. doi:10.1111/evo.12632
Gossard TW, Jones RE (1977) Effects of age and weather on egg-laying in *Pieris rapae L* Journal of Applied Ecology 14 (1):65-71. doi:10.2307/2401827
Gross K, Sun M, Schiestl FP (2016) Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. Plos One 11 (2):e0147975-e0147975. doi:10.1371/journal.pone.0147975
Herrera CM, Medrano M, Rey PJ, Sanchez-Lafuente AM, Garcia MB, Guitian J, Manzaneda AJ (2002) Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. Proceedings of the National Academy of Sciences of the United States of America 99 (26):16823-16828. doi:10.1073/pnas.252362799
Honda K, Omura H, Hayashi N (1998) Identification of floral volatiles from Ligustrum japonicum that stimulate flower-visiting by CABBAGE butterfly, Pieris rapae. Journal of Chemical Ecology 24 (12):2167-2180. doi:10.1023/a:1020750029362
Jones RE, Hart JR, Bull GD (1982) Temperature, size and egg-production in the cabbage butterfly, *Pieris rapae* L Australian Journal of Zoology 30 (2):223-232. doi:10.1071/zo9820223
Juenger T, Bergelson J (1998) Pairwise versus diffuse natural selection and the multiple herbivores of scarlet gilia, Ipomopsis aggregata. Evolution 52 (6):1583-1592. doi:10.2307/2411332
Kessler A, Halitschke R (2009) Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. Functional Ecology 23 (5):901-912. doi:10.1111/j.1365-2435.2009.01639.x
Knauer AC, Schiestl FP (2015) Bees use honest floral signals as indicators of reward when visiting flowers. Ecology Letters 18 (2):135-143. doi:10.1111/ele.12386
Knight TM, Steets JA, Vamosi JC, Mazer SJ, Burd M, Campbell DR, Dudash MR, Johnston MO, Mitchell RJ, Ashman TL (2005) Pollen limitation of plant reproduction: Pattern and process. In: Annual Review of Ecology Evolution and Systematics, vol 36. Annual Review of Ecology Evolution and Systematics. pp 467-497. doi:10.1146/annurev.ecolsys.36.102403.115320
Kudoh H, Whigham DF (1998) The effect of petal size manipulation on pollinator/seed-predator mediated female reproductive success of Hibiscus moscheutos. Oecologia 117 (1-2):70-79. doi:10.1007/s004420050633
Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. Evolution 37 (6):1210-1226

McCall AC, Irwin RE (2006) Florivory: the intersection of pollination and herbivory. Ecology Letters 9 (12):1351-1365. doi:10.1111/j.1461-0248.2006.00975.x
McCall AC, Murphy SJ, Venner C, Brown M (2013) Florivores prefer white versus pink petal color morphs in wild radish, *Raphanus sativus*. Oecologia 172 (1):189-195. doi:10.1007/s00442-012-2480-z
Medel R, Botto-Mahan C, Kalin-Arroyo M (2003) Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. Ecology 84 (7):1721-1732. doi:10.1890/01-0688
Omura H, Honda K, Hayashi N (1999) Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. Journal of Chemical Ecology 25 (8):1895-1906. doi:10.1023/a:1020990018111
Parachnowitsch AL, Caruso CM (2008) Predispersal seed herbivores, not pollinators, exert selection on floral traits via female fitness. Ecology 89 (7):1802-1810. doi:10.1890/07-0555.1
Rader R, Howlett BG, Cunningham SA, Westcott DA, Newstrom-Lloyd LE, Walker MK, Teulon DAJ, Edwards W (2009) Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. Journal of Applied Ecology 46 (5):1080-1087. doi:10.1111/j.1365-2664.2009.01700.x
Raguso RA (2008) Wake up and smell the roses: The ecology and evolution of floral scent. Annual Review of Ecology Evolution and Systematics 39:549-569. doi:10.1146/annurev.ecolsys.38.091206.095601
Rey PJ, Herrera CM, Guitian J, Cerda X, Sanchez-Lafuente AM, Medrano M, Garrido JL (2006) The geographic mosaic in predispersal interactions and selection on Helleborus foetidus (Ranunculaceae). Journal of Evolutionary Biology 19 (1):21-34
Reynolds RJ, Kula AAR, Fenster CB, Dudash MR (2012) Variable nursery pollinator importance and its effect on plant reproductive success. Oecologia 168 (2):439-448. doi:10.1007/s00442-011-2095-9
Root RB (1973) Organization of a plant-arthropod association in simple and diverse habitats - fauna of collards (*Brassica oleracea*) Ecological Monographs 43 (1):95-120. doi:10.2307/1942161
Rosas-Guerrero V, Aguilar R, Marten-Rodriguez S, Ashworth L, Lopezaraiza-Mikel M, Bastida JM, Quesada M (2014) A quantitative review of pollination syndromes: do floral traits predict effective pollinators? Ecology Letters 17 (3):388-400. doi:10.1111/ele.12224
Roubik DW (1978) Competitive interactions between neotropical pollinators and africanized honey bees Science 201 (4360):1030-1032. doi:10.1126/science.201.4360.1030
Sahli HF, Conner JK (2007) Visitation, effectiveness, and efficiency of 15 genera of visitors to wild radish, Raphanus raphanismum (Brassicaceae). American Journal of Botany 94 (2):203-209. doi:10.3732/ajb.94.2.203
Sahli HF, Conner JK (2011) Testing for conflicting and nonadditive selection: Floral adaptation to multiple pollinators through male and female fitness. Evolution 65 (5):1457-1473. doi:10.1111/j.1558-5646.2011.01229.x
Sanchez-Lafuente AM (2007) Corolla herbivory, pollination success and fruit predation in complex flowers: An experimental study with Linaria lilacina (Scrophulariaceae). Annals of Botany 99 (2):355-364. doi:10.1093/aob/mcl267
Schemske DW, Bradshaw HD (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (Mimulus). Proceedings of the National Academy of Sciences of the United States of America 96 (21):11910-11915. doi:10.1073/pnas.96.21.11910
Schiestl FP, Huber FK, Gomez JM (2011) Phenotypic selection on floral scent: trade-off between attraction and deterrence? Evolutionary Ecology 25 (2):237-248. doi:10.1007/s10682-010-9409-y
Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28 (5):307-315. doi:10.1016/j.tree.2013.01.019
Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. New Phytologist 203 (1):257-266
Siepielski AM, Benkman CW (2010) Conflicting selection from an antagonist and a mutualist enhances phenotypic variation in a plant. Evolution 64 (4):1120-1128
Sletvold N, Agren J (2010) Pollinator-mediated selection on floral display and spur length in the orchid *Gymnadenia conopsea* International Journal of Plant Sciences 171 (9):999-1009. doi:10.1086/656597
Sletvold N, Moritz KK, Agren J (2015) Additive effects of pollinators and herbivores result in both conflicting and reinforcing selection on floral traits. Ecology 96 (1):214-221. doi:10.1890/14-0119.1

Smallegange RC, van Loon JJA, Blatt SE, Harvey JA, Agerbirk N, Dicke M (2007) Flower vs. leaf feeding by Pieris brassicae: Glucosinolate-rich flower tissues are preferred and sustain higher growth rate. Journal of Chemical Ecology 33 (10):1831-1844. doi:10.1007/s10886-007-9350-x

Stadler E, Renwick JAA, Radke CD, Sachdevgupta K (1995) Tarsal contact chemoreceptor response to glucosinolates and cardenolides mediating oviposition in *Pieris rapae* Physiological Entomology 20 (2):175-187. doi:10.1111/j.1365-3032.1995.tb00814.x

Strauss SY, Irwin RE (2004) Ecological and evolutionary consequences of multispecies plant-animal interactions. Annual Review of Ecology Evolution and Systematics 35:435-466. doi:10.1146/annurev.ecolsys.35.112202.130215

Strauss SY, Whittall JB (2006) Non-pollinator agents of selection on floral traits. Ecology and evolution of flowers:120-138

Theis N (2006) Fragrance of canada thistle (*Cirsium arvense*) attracts both floral herbivores and pollinators. Journal of Chemical Ecology 32 (5):917-927. doi:10.1007/s10886-006-9051-x

Theis N, Adler LS (2012) Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. Ecology 93 (2):430-435

Theis N, Barber NA, Gillespie SD, Hazzard RV, Adler LS (2014) Attracting mutualists and antagonists: plant trait variation explains the distribution of specialist floral herbivores and pollinators on crops and wild gourds. American Journal of Botany 101 (8):1314-1322. doi:10.3732/ajb.1400171

Tholl D, Boland W, Hansel A, Loreto F, Rose USR, Schnitzler JP (2006) Practical approaches to plant volatile analysis. Plant J 45 (4):540-560. doi:10.1111/j.1365-313X.2005.02612.x

Thompson JN, Cunningham BM (2002) Geographic structure and dynamics of coevolutionary selection. Nature 417 (6890):735-738. doi:10.1038/nature00810

Thomson D (2004) Competitive interactions between the invasive European honey bee and native bumble bees. Ecology 85 (2):458-470. doi:10.1890/02-0626

Valterova I, Kunze J, Gumbert A, Luxova A, Liblikas I, Kalinova B, Borg-Karlson AK (2007) Male bumble bee pheromonal components in the scent of deceit pollinated orchids; unrecognized pollinator cues? Arthropod-Plant Interactions 1 (3):137-145. doi:10.1007/s11829-007-9019-y

Watanabe M, Ito A, Takada Y, Ninomiya C, Kakizaki T, Takahata Y, Hatakeyama K, Hinata K, Suzuki G, Takasaki T, Satta Y, Shiba H, Takayama S, Isogai A (2000) Highly divergent sequences of the pollen self-incompatibility (S) gene in class-I S haplotypes of Brassica campestris (syn. rapa) L. Febs Lett 473 (2):139-144. doi:10.1016/s0014-5793(00)01514-3

Wise MJ, Hebert JB (2010) Herbivores affect natural selection for floral-sex ratio in a field population of horsenettle, *Solanum carolinense*. Ecology 91 (4):937-943. doi:10.1890/09-1373.1

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenson J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proceedings of the National Academy of Sciences of the United States of America 101 (14):4859-4864. doi:10.1073/pnas.0308007101

Zu P, Blanckenhorn WU, Schiestl FP (2016) Heritability of floral volatiles and pleiotropic responses to artificial selection in *Brassica rapa*. The New phytologist 209 (3):1208-1219. doi:10.1111/nph.13652

SUPPORTING INFORMATION

Effect of infestation on plant fitness

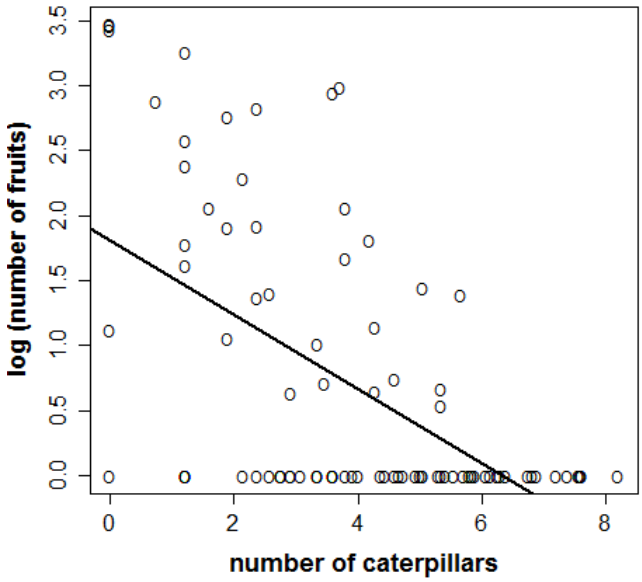


Figure S1 Regression between number of fruits and number of caterpillars in infested plants. The number of caterpillars had a significant negative effect on the number of fruits that developed (estimate ± s.e. = 0.29 ± 0.05, t = -6.253, P < 0.001). We used BoxCox-transformation of the number of caterpillars to obtain homogeneity of variance, and approach normal distributions.

Variance of floral scent compounds

Table S4 Mean amounts of floral scent compounds in pg/(l*flower), their standard deviation and coefficient of variance.

Floral scent compound	mean ± s.e.	s.d.	c.v.
Benzaldehyde	829 ± 37	698	0.84
1-Butene-4-isothiocyanate	171 ± 15	285	1.67
Phenylacetaldehyde	2180 ± 139	2646	1.21
Methyl benzoate	131 ± 7	130	1.00
Methyl salicylate	45 ± 3	50	1.10
p-Anisaldehyde	137 ± 8	161	1.17
(*E*)-α-Farnesene	2130 ± 56	1070	0.50

Floral Scent Compounds

Table S1 Mean emission (pg/(l*flower)) of all floral scent compounds (N = 60).

Compound	Mean ± s.e.
Aromatic compounds	
Benzaldehyde	846.2 ± 41.2
Phenylacetaldehyde	2273.5 ± 156.3
Methyl benzoate	130.8 ± 6.9
Phenylethyl alcohol	128.4 ± 10.0
Methyl salicylate	45.1 ± 2.6
2-Aminobenzaldehyde	331.8 ± 23.0
p-Anisaldehyde	140.8 ± 9.2
Phenylethyl acetate	3.0 ± 0.5
Terpenoids	
β-Pinene	54.3 ± 1.1
Limonene	83.5 ± 3.5
Linalool	53.6 ± 2.7
(Z)-α-Farnesene	137.3 ± 6.6
(E)-α-Farnesene	2145.7 ± 58.8
Fatty acid derivatives	
(Z)-3-Hexenyl acetate	463.7 ± 25.4
Nitrogen containing compounds	
Benzyl nitrile	39.7 ± 3.7
Indole	46.5 ± 3.2
Methylanthranilate	14.8 ± 1.4
Sulphur containing compound	
1-Butene-4-isothiocyanate	170.7 ± 15.0

Test for nonadditive selection

To test for nonadditive selection we generated a predicted dataset by predicting the relative fitness from a multivariate model with the calculated additive selection gradients and the floral trait values from pollinator treatment c) (bumble bees and cabbage butterflies). Finally, we merged the predicted dataset with the actual one and fitted a model with *relative fitness* as response and the *floral traits* and the *data set* (predicted vs actual data) as explanatory variables. A significant interaction between the *data set* and the *floral traits* indicates nonadditive selection among pollinators.

Correlation between floral and vegetative traits

Table S2 Correlations between floral and vegetative traits. Corolla size and the floral volatile phenylacetaldehyde attracted cabbage butterflies to oviposit on leaves.

	Floral trait																	
	Flower nr.		Corolla size		Benzaldehyde		Phenylacetald.		1-But.-4-isothio.		Methyl benzoate		Methyl salicilate		p-Anisaldehyde		(*E*)-α-Farnes.	
Vegetative trait	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
Plant height	-0.31	0.08	0.13	0.46	-0.30	0.09	0.06	0.74	-0.04	0.84	-0.39	0.026	-0.40	**0.020**	-0.22	0.22	-0.04	0.85
Leaf number	-0.39	**0.025**	0.17	0.36	-0.19	0.29	0.06	0.73	0.19	0.30	0.01	0.96	0.04	0.83	-0.31	0.08	0.06	0.77
Glucobrassicin	0.21	0.31	-0.11	0.61	0.26	0.21	-0.32	0.11	-0.15	0.46	-0.05	0.82	0.16	0.44	0.45	**0.023**	-0.10	0.62
Gluconapin	-0.31	0.13	-0.02	0.92	0.01	0.96	-0.52	**0.007**	-0.10	0.63	-0.13	0.52	-0.18	0.40	-0.10	0.62	-0.28	0.17
Hydroxyglucobrassicin	0.40	**0.048**	0.09	0.66	0.13	0.54	-0.26	0.21	-0.08	0.70	-0.16	0.45	-0.01	0.95	0.25	0.22	-0.12	0.57
Glucoraphanin	0.01	0.96	-0.31	0.13	-0.07	0.72	-0.2	0.34	-0.37	0.07	-0.06	0.77	0.21	0.31	-0.13	0.53	-0.17	0.41
Methoxyglucobrassicin	-0.07	0.72	-0.22	0.29	-0.04	0.84	0.03	0.88	0.23	0.27	0.23	0.27	0.03	0.87	-0.11	0.60	0.39	0.05
Neoglucobrassicin	0.02	0.93	0.12	0.58	-0.36	0.08	-0.32	0.12	0.45	**0.025**	-0.42	**0.035**	-0.16	0.43	-0.29	0.15	-0.32	0.12
Glucobrassicanapin	0.10	0.64	-0.24	0.26	0.01	0.96	-0.33	0.11	-0.09	0.68	-0.18	0.40	-0.12	0.58	0.23	0.27	-0.07	0.73
Glucoerucin	0.16	0.46	0.13	0.54	0.18	0.40	-0.09	0.68	-0.03	0.87	0.08	0.71	0.09	0.68	0.14	0.50	-0.06	0.75
Gluconasturciin	0.31	0.13	0.17	0.41	0.01	0.95	-0.17	0.40	0.06	0.78	-0.05	0.80	0.02	0.94	-0.03	0.90	0.07	0.72

Correlation between floral traits

Table S3 Correlations between floral traits. Bonferroni correction was used to control for multiple testing.

	Floral trait															
	Corolla size		Benzaldehyde		Phenylacetald.		1-But.-4-isothio.		Methyl benzoate		Methyl salicylate		p-Anisaldehyde		(*E*)-α-Farnesene	
Floral trait	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
Flower number	0.15	0.14	-0.04	1	0.13	0.58	-0.14	0.22	0.11	1	0.03	1	-0.01	1	-0.10	1
Corolla size	-	-	0.03	1	0.09	1	-0.08	1	0.1	1	0.12	0.90	0.08	1	0.05	1
Benzaldehyde	-	-	-	-	0.38	**< 0.001**	0.15	0.18	0.41	**< 0.001**	0.19	**0.014**	0.82	**< 0.001**	0.40	**< 0.001**
Phenylacetald.	-	-	-	-	-	-	0.12	0.720	0.12	0.65	0.21	**< 0.001**	0.45	**< 0.001**	0.32	**< 0.001**
1-But.-4-isothio.	-	-	-	-	-	-	-	-	-0.02	1	-0.04	1	0.13	0.47	0.06	1
Methyl benzoate	-	-	-	-	-	-	-	-	-	-	0.56	**< 0.001**	0.39	**< 0.001**	0.29	**< 0.001**
Methyl salicylate	-	-	-	-	-	-	-	-	-	-	-	-	0.26	**< 0.001**	0.16	0.11
p-Anisaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.40	**< 0.001**

CHAPTER IV

Camouflage uncovered: the disclosed effect of crab spiders on floral-signal evolution

Knauer, A.C.[1] , Bakhtiari M.[1,2] and Schiestl, F.P.[1*]

[1] Department of Systematic and Evolutionary Botany, Zollikerstrasse 107, University of Zurich, 8008 Zurich, Switzerland

[2] current address: *Institute of Biology, Rue Emile-Argand 11, University of Neuchatel, 2000 Neuchatel, Switzerland*

Contribution statement

Mojtaba Bakhtiari conducted the behavioral assays testing for the crab spider's preference for pure scent compounds. Anina C. Knauer collected the remaining data.

ABSTRACT

The puzzling diversity of flowers is thought to be primarily shaped by the plant's interaction with animals, causing selection and evolutionary change. The effect of individual animal species on net selection may vary depending on the network of interacting organisms. For example, flower-dwelling predators like crab spiders can harm plants by hunting pollinators, but also benefit them by feeding on herbivores. Here we document that the buckler mustard, *Biscutella laevigata*, experiences a conflict between pollinator- and predator attraction. The crab spider *Thomisus onustus* displaces bees from flowers, but shares a preference with bees for the floral volatile β-ocimene. In florivore-infested plants, however, crab spiders largely feed on florivores increasing plant fitness. Plants infested with florivores show an induced emission of β-ocimene, which is stronger in plant populations where crab spiders are present than where they are absent. Crab spiders also preferred plants from populations with spiders, but only after florivore infestation, suggesting plants are locally adapted to the presence of crab spiders. Our study demonstrates context-dependence of the selection on floral traits imposed by individual plant-animal interactions and discloses the rarely considered relevance of crab spiders for floral signal evolution.

INTRODUCTION

Plant-animal interactions are a major source of selection on plant traits, driving plant evolution, local adaptation and species divergence (van der Niet and Johnson 2012; Thompson et al. 2013; Thompson and Cunningham 2002; Sun et al. 2014). Most plants interact with various mutualists (e.g. pollination) and antagonists (e.g. herbivory or florivory) directly (Thompson and Cunningham 2002; Bronstein et al. 2006; Strauss and Irwin 2004; Hoeksema and Bruna 2015), but may also interact indirectly with animals from the third trophic level (e.g. predators or parasitoids) (Heil 2008). The fitness outcome of such indirect plant-animal interaction depends on whether the involved animals hunt mostly on mutualists or antagonists. However, with all these animal interactors plants can communicate via floral signals (Schaefer and Ruxton 2011). Signals that attract mutualists but deter antagonists will be favored by natural selection. Selection on plant traits, however, is shaped not only by pairwise interactions but by the entire network of interacting organisms (Gomez 2005; Gómez 2008; Gomez 2003; Rey et al. 2006), as the fitness effect through one interaction can depend on the presence or intensity of another interaction (Strauss and Irwin 2004; Armbruster 1997). For example, many floral signals have evolved to attract mutualists, mostly pollinators (Schiestl and Johnson 2013), but antagonists can eavesdrop on such signals and use them to their own advantage. Such eavesdropping can cause a trade-off between the attraction of mutualists and the avoidance of antagonists (Theis and Adler 2012; Schiestl et al. 2011; Brody and Mitchell 1997) and result in conflicting selection on the same plant trait (Gomez 2003; Gómez 2008). Further, the presence of one interaction can affect the fitness outcome of another interaction (Strauss and Irwin 2004). For example, the same predator may affect plant fitness differently when feeding on plant mutualists than when feeding on plant antagonists depending on their relative abundance.

Crab spiders use camouflage on flowers to hunt for flower-visiting insects such as pollinators (Figure 1) (Heiling et al. 2005) and are thus thought to have a negative effect on plant fitness (Antiqueira and Romero 2016; Goncalves-Souza et al. 2008; Dukas 2001). Both crab spiders and bees are attracted to flowers by scent (Heiling et al. 2004), suggesting an eavesdropping scenario in which crab spiders exploit pollinator-attracting signals to find prey. However, because flower scent usually comprises complex blends of volatile compounds, it is still unknown whether spiders use the same volatiles than pollinators to find flowers, and whether plants experience a conflict between the attraction of bees and the avoidance of crab spiders. Further, crab spiders are generalist predators and sometimes also feed on insect herbivores (Figure 1), a phenomenon that can reduce flower damage and increase plant fitness (Romero and Vasconcellos-Neto 2004; Gonzalez et al. 2009). Mathematical modelling predicted that the net effect of generalist flower-dwelling predators on plant fitness can be positive or negative depending on the relative abundance of mutualists and antagonists in a plant population (Higginson et al. 2010). However, experimental evidence for this is lacking, and the net effects of crab spiders on plant fitness in natural environments has not yet been analyzed. In this study we investigate the effect of crab spiders on the plant's interaction with pollinators and florivores and the consequences for floral trait evolution in the alpine herb *Biscutella laevigata* (Figure 1). In the Swiss and Italian valleys where this study was conducted the crab spider *Thomisus onustus* occurs in lowland populations on up to 30% of plants, but is absent from highland populations. Plant populations within each valley share evolutionary origin and belong to the same genetic lineage (Parisod and Besnard 2007). In the spider-associated populations, plants are mainly pollinated by bees and florivore-infestation rates can reach 40% of plants. Using this study system we address the following specific questions: 1) Does *B. laevigata* experience a conflict

between the attraction of pollinators and the avoidance of crab spiders? 2) How does the presence of crab spiders (*Thomisus onustus*) affect pollinator-mediated selection on floral traits? 3) What is the fitness effect of *T. onustus* on *B. laevigata* in the absence or presence of florivores? 4) Does *B. laevigata* induce a crab spider-attracting scent compound when infested with florivores?; 5) Are *B. laevigata* populations locally adapted in their indirect defense to the presence of the crab spiders?

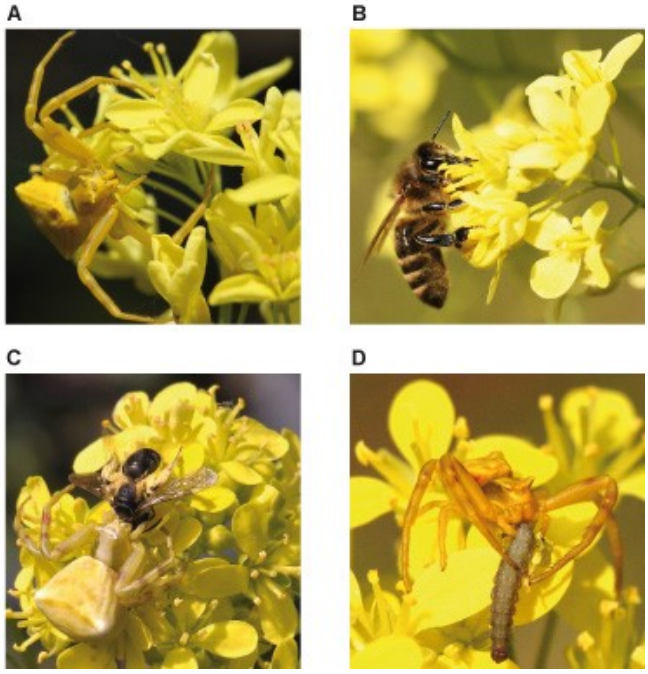


Figure 1: The buckler mustard (*Biscutella laevigata*) and various interacting organisms. *(A)* A crab spider (*Thomisus onustus*) on *B. laevigata* flowers. *(B)* A honey bee (*Apis mellifera*) visiting *B. laevigata* flowers. *(C)* A crab spider feeding on a wild bee (*Halictus sp.*) *(D)* A crab spider feeding on a florivore *(Plutella xylostella).*

MATERIALS AND METHODS

Study organism

Biscutella laevigata ssp laevigata (Brassicaceae) is a perennial herb native to the central parts of the Alps, where it occurs from about 500 to over 2000 m.a.s.l.. *B. laevigata* is self-incompatible (Olowokudejo and Heywood 1984) and has a generalized pollination system with a large variety of pollinators from the orders Hymenoptera, Diptera, Lepidoptera and Coleoptera (Figure 1: Table S1). *B. laevigata* flowers are attacked by various different species of florivores, among which *Plutella xylostella*, various species of Pieridae, and different species of Coleopterans are some of the most frequently found (personal observation). The floral scent of *B. laevigata* is strongly dominated by only three scent compounds: the monoterpene β-ocimene (E- and Z-isomere, ratio about 1:3) and the two aromatics p-anisaldehyde and 2-aminobenzaldehyde.

We conducted our study in four populations of *B. laevigata*, two in Switzerland and two in Italy. Although they belong to the same tetraploid subspecies, populations in the lower Rhone valley, Switzerland, belong to a different lineage than the populations in the Aosta valley, Italy, indicating different evolutionary origins (Parisod and Besnard 2007). We selected a lowland and a highland population in each valley (Swiss lowland: 46° 07’ 53’’ N, 7° 03’ 51’’ E, 500 m.a.s.l.; Swiss highland: 46° 01’ 36’’ N, 7° 28’ 53’’ E, 2000 m.a.s.l.; Italian lowland: 45° 42’ 12’’ N, 7° 12’ 38’’; 700 m.a.s.l.; Italian highland: 45° 49’ 21’’ N; 7° 37’ 10’’ E; 1950 m.a.s.l.). Whereas lowland populations are predominately pollinated by bees, at high elevations various fly species and a sawfly dominate the pollinator guilds (see SI Appendix for characterization of the pollinator guilds, Table S1 and S2). Further, both lowland populations are associated with the crab spider *Thomisus onustus* (Figure 1), whereas no spiders were found in both highland populations. *T. onustus* has two

reproductive periods in spring and autumn and therefore depend on extended flowering seasons making the alpine highlands an unsuitable habitat (Levy 1970). In both lowland populations crab spiders hunt insects nearly exclusively on *B. laevigata* flowers (personal observation). Spider abundance varies strongly within the flowering season and can reach values of up to 30% of the plants being occupied by them (see SI Appendix for more detailed information). Little is known about any preferences of *T. onustus* for floral signals, but the related species *Thomisus spectabilis* has been shown to be attracted to flowers mainly by floral scent (Heiling et al. 2004).

For all experiments we used plants cultivated in a common garden environment from seeds collected from wild plants. *B. laevigata* seeds were collected from 50 individuals in each of the four populations, except for the Italian highland population where only 20 individuals with ripe fruits could be found. The seeds were germinated and experimental plants were grown outdoors in the Botanical Garden of Zurich. All plants were kept under netting before the start of flowering to avoid pollination and infestation with florivores until experimental use. For the *Local Adaptation* experiment we used seeds from all four populations. The other experiments were conducted with plants cultivated from Swiss lowland seeds.

Experiments with pollinators and crab spiders were conducted on a meadow about 50 m from the Swiss lowland population. This allowed us to work with the natural pollinator guild and to collect crab spiders directly from *B. laevigata* flowers in the natural population. Only female spiders were used in the experiment as they are the main foragers and are two to three times bigger than males (Levy 1970; Roberts 1996). All experiments were performed during the flowering season of *B. laevigata* in spring 2015 and 2016. For experimental florivore infestations we used larvae of the diamondback moth *Plutella xylostella* (obtained from Syngenta, Stein AG,

Switzerland), which is a common florivore species in Brassicaceae (Reed et al. 1989).

Interaction between crab spider and pollinator attraction

Spider attraction

To determine which scent compound(s) attract *T. onustus* to *B. laevigata* flowers we conducted dual choice behavioral assays. Each of the three scent compounds was tested against an odorless control using a Y-tube olfactometer (15 cm arm length, 1 cm diameter, 45° angle; n = 30 *T. onustus* individuals per compound). Scent compounds were emitted from grey rubber septa (Supleco, Bellefonte, PA, USA) at emission rates similar to those of one *B. laevigata* individual. Septa were soaked for one hour in a solution of synthetic scent compounds in solvent and subsequently dried for about 2 hrs to reach constant emission rates (see SI Appendix for details about scent solutions; emission rates from septa and plants are given in Table S3). Odorless control septa were soaked in pure solvent. Scented and control septa were placed at the two ends of the Y-tube. Each end was connected to a membrane pump (Personal Air Sampler, SKC, USA), which pushed air into the tube at a rate of 150 ml min^{-1}. After each choice by a spider the Y-tube was cleaned with acetone and water and the positions of the two septa were exchanged. All behavioral assays were analyzed using binomial tests. The proportion of choices for one category was tested against an expected value of 0.5.

As crab spiders showed a strong preference for β-ocimene in the dual-choice behavioral assays, we then tested for an association between the emission of this compound and the presence of crab spiders in the natural *B. laevigata* population (Swiss lowland). We collected scent from the inflorescences of 94 randomly selected individuals (volatile sampling and analysis as described below), which were in full

flower. For each of these individuals we additionally recorded if there was a female *T. onustus* on the inflorescence or not (33 individuals with spider and 69 without). To test for an association between spider distribution and β-ocimene emission we used a generalized linear model with binomial distribution. The presence of spiders (absent vs. present) was fitted as the response and the amount of β-ocimene as explanatory variable.

Bee attraction

To test for a conflict between pollinator attraction and predator avoidance in *B. laevigata*, we measured whether β-ocimene was also attractive to bees. To do so we conducted a dual-choice behavioral assay presenting bees in the field a choice between a plant with augmented β-ocimene emission and a plant with unmanipulated emission (control). The two plants were placed 20 cm apart on the meadow and each alight of a bee on an inflorescences was recorded. In total we observed 34 landings; after each landing the position of plants was exchanged. Scent was augmented by fixing a rubber septa emitting β-ocimene on the inflorescence, control plants received odorless septa (septa preparation as described above). Scented and odorless septa were exchanged between plants after half of the landings. Data were analyzed using a binomial test. The proportion of choices for one category was tested against an expected value of 0.5.

Effect of crab spiders on plant-pollinator interaction

We used field plot experiments to investigate the influence of crab spiders on pollinator behavior and pollinator-mediated selection on floral traits. Six plots of 36 *B. laevigata* plants (6 x 6 plants, 40 cm distance between plants) were placed in the field for five sunny days under one of two treatments: *a) no crab spiders in the plot; b) 9 to*

12 crab spiders hunting in the plot. For each treatment three replicates were conducted leading to a sample size of 108 plants per treatment. For all plants used in this experiment we measured floral scent and flower size (measurement of floral traits described below) one day before the plots were placed at the field site. These measurements were conducted in the greenhouse under standardized light and temperature conditions. Additionally, to count flower number, we marked the lowest flower on the first day and then counted the pedicles from markings to buds at the last day of the experiment. Pollinators were observed during 17.5 h, with each plot observed for about 5 minutes before moving to the next plot. We only noted visits by bees (Apidae and Halictidae) as they were the dominant pollinators in our field site with respect to abundance and pollinator effectiveness (Table S1 and S2). In treatment b) spiders were allowed to move freely inside the plots. Spider abundance was monitored regularly and whenever the number of spiders hunting on flowers dropped below 9 a new individual was released. The position of each crab spider inside the plots was noted three times a day. For each *B. laevigata* individual we quantified the abundance of crab spiders as the sum of time intervals in which a spider was observed on the inflorescence. To measure pollinator-mediated selection we used the number of visits by bees as a plant fitness estimate. Floral traits were standardized by z-transformation within plots for statistical analysis. To test for differences of selection gradients between treatments, we used a generalized mixed effect model with poisson distribution. The number of visits by bees was included as the response, floral traits and treatment as explanatory variables, and plot as a random effect. Subsequently we used analysis of variance for the model objects produced by a generalized mixed effect model to test for significant treatment x trait interactions, which indicate differences in the selection gradients between treatments. To calculate selection gradients on the floral traits for which a significant trait x

treatment interaction was found, we fitted a generalized mixed effect model with poisson distribution for each treatment separately. To measure selection gradients on the traits that did not show a treatment x trait interaction, we fitted a model with the data from both treatments. In these models the number of visits by bees was included as the response, floral traits as explanatory variables and plot as a random effect. Further, to test for the direct effect of crab spiders on bee attraction we included the spider abundance as explanatory variable in the model (for treatment b) only).

Tritrophic interaction

Effect of crab spiders on plant fitness

We tested for antagonistic and mutualistic effects of crab spiders on plant fitness in the absence and presence of florivores. 25 plots of 4 plants each (20 cm distance between plants) were placed in the field for two to four days (depending on the weather). Within each plot, individuals were randomly assigned to one of the following treatments: *a) control; b) a crab spider on inflorescence; c) infestation with florivores; d) a crab spider on inflorescence and infestation with florivores.* Every morning we placed 3 *P. xylostella* larvae (larval stages L2 to L3) on the plants in treatments c) and d) and counted the remaining number of larvae in the evening. In these treatments, we additionally counted the number of damaged flowers and buds on the last experimental day to quantify total floral damage by florivores. Three times a day we controlled the position of spiders in the plots and returned them to the right plants if necessary. Additionally we noted which spiders were feeding on a prey, and the type of prey (pollinator or *P. xylostella* larvae). About four weeks after the experiment we counted the number of developed seeds. Because *B. laevigata* plants can develop a maximum of two seeds from each flower, we calculated the individual

fitness as *(number of seeds)/(2*number of open flowers during experiment)* to control for differences in flower number between plants. We used a linear mixed model to analyze the effect of crab spiders and florivory on plant fitness. Spider presence (present vs. absent) and florivore infestation (present vs. absent) were fitted as explanatory variables and plot was included as a random effect. Linear mixed models were also used to analyze the effect of spider presence on the number of florivores on the inflorescence and floral damage. The spider presence (present vs. absent) was fitted as explanatory variable and plot as random effect in both analyses.

Because spiders had a positive effect on plant fitness in florivore-infested plants, we quantify the proportion of florivores in the diet of crab spiders in the natural *B. laevigata* population. We searched all plants in the population for crab spiders and identified all their prey as florivores (caterpillars) or pollinators (bees or syrphid flies) during 5 days distributed over the whole flowering season. In total we found 17 spiders with a prey. Further, to test if crab spiders prefer hunting on florivore-infested plants, we scanned 105 plants for the presence of florivores and crab spiders on inflorescences. To analyze this data we used a binomial test and tested the proportion of infested plants that carried a crab spider against the proportion of infested plants in the whole population as expected value.

Effect of florivore infestation on floral scent

To test for the inducibility of the spider attractant β-ocimene by florivory we grew 68 plants from 34 half-sib families (2 plants per family, seeds from Swiss lowland population). Individuals from the same family were assigned randomly to one of two treatments: *a) control; b) infestation with P. xylostella larvae*. We quantified constitutive volatile emission in all plants before infestation (volatile sampling and analysis as described below). The next day, plants in treatment b) were infested with

larvae in stage L2 and about 24 hours afterwards the scent collection was repeated. Measurements were made over three consecutive weeks, and we sampled the same number of plants per treatment each week. In total we measured 28 plants per treatment (from 6 families we could only measure one individual). Scent collection was conducted in the greenhouse under standardized light and temperature conditions. To measure inducibility of β-ocimene, the change in sent emission from the first to the second scent collection was calculated in percentage change per flower. This variable was the response in a linear mixed model with treatment (infested vs. control) as an explanatory variable and the half-sib family as a random effect. To approach normal distribution of residuals, the response was Box-Cox transformed with a lambda of 0.7 for statistical analysis.

Attraction of crab spiders by florivore-infested plants

To test whether crab spiders showed a preference for florivore-infested plants, we used a dual-choice behavioral assay. We presented 29 crab spiders with pairs of plants with similar flower number within a pair (maximal difference of 8 flowers, 15 pairs in total). One plant per pair was selected randomly and infested with 5 *P. xylostella* larvae (stage L2) two days before the behavioral assay, while the other plant (control) was left uninfested. Plants were presented to crab spiders at a distance of 20 cm. The positions of the infested and the control plant were switched after each trial to control for wind direction and light conditions. Crab spiders were placed on a wooden stick in the middle of the two plants. The top of the stick was at flower level (at the middle when plants had different inflorescence heights) allowing crab spiders to directly move to inflorescences. The first plant they moved to and settled on flowers was noted as their choice. Data were analyzed using a binomial

test. The proportion of choices for one category was tested against an expected value of 0.5.

Local Adaptation to crab spiders

Inducibility of β-ocimene

To test for differences in the inducibility of β-ocimene between low- and highland *B. laevigata* populations we quantified volatile emission in plants before and after infestation with *P. xylostella* larvae. For the Swiss lineage 30 plants per population were sampled, for the Italian lineage 25. On one day, we collected scent from the same number of plants from the low- and highland populations per region (volatile sampling and analysis as described below). The next day, all plants were infested with 5 *P. xylostella* larvae (in stage L2) and about 24 hours after infestation the volatile sampling was repeated. Scent collection was conducted in the greenhouse under standardized light and temperature conditions. Inducibility of β-ocimene was calculated as difference in the emitted amount per flower before and after florivore infestation. We fitted a linear model with the altitude (low- vs. highland) and the region (Swiss vs. Italian) as explanatory variables to test for differences in inducibility due to differences in spider occurrence and region, respectively. Additionally, the absolute emission values before and after infestation were analyzed with the same model.

Behavioral assays

Because we found a positive fitness effect of crab spiders on infested plants we investigated local adaptation to crab spiders by testing for an increased attractiveness of lowland and highland plants after florivore infestation. We used dual-choice behavioral assays; each spider was presented with one lowland and one

highland plant from the same region (Italy or Switzerland). Two assays were conducted; one with infested plants and one with control plants. Plants were infested with 5 *P. xylostella* larvae (stage L2) two days before the behavioral assay. During the assays lowland and highland plants were presented to crab spiders with a distance of 20 cm, and the positions of the two plants were switched after each trial to control for wind direction and light conditions. Crab spiders were placed on a wooden stick in the middle of the two plants. The top of the stick was at flower level (at the middle when plants had different inflorescence heights) allowing crab spiders to directly move to inflorescences. The first plant they moved to and settled on flowers was noted as their choice. For each comparison (Italian and Swiss region, infested and control) we tested 19 to 24 pairs of plants. Behavioral assays were analyzed using binomial tests. The proportion of choices for one category was tested against an expected value of 0.5.

Measurement of floral traits

For scent collection in the field we used the dynamic headspace collection method (Gross et al. 2016). Inflorescences were inserted into polyethylene terephtalate cooking bags which were closed around the stem with a wire. Glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into the bag from the other side and attached to a Micro Air Sampler (PAS-500 Micro Air Sampler, Spectrex, Redwood City, CA, USA) with a silicon tube. Air was pulled through the Tenax tubes at a flow rate of 150 ml min^{-1}. All collections took place between 11^{00} and 15^{00}hrs. After scent collection the Tenax tubes were stored in sealable glass tubes (7 ml, Sigma-Aldrich, Buchs, Switzerland).

For scent collection from inflorescences in the greenhouse we used the push-pull headspace collection method (Schiestl et al. 2014). Inflorescences were

enclosed in glass cylinders (dimensions: 5 cm diameter, 25 cm height; all glass cylinders were treated previously with sigmacoate (Sigma-Aldrich, Buchs, Switzerland)). The bottom of the cylinder was closed with a teflon plate with a central hole allowing for the insertion of the peduncle without injuring it. For volatile collection glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into a small opening in the cylinder and attached to a vacuum pump (DC06/04/20F, Fürgut GmbH, D-88459 Tannheim) with a silicon tube. Air was pulled through the Tenax tubes at a flow rate of 150 ml min^{-1}. After passing the tube, the air was circulated back (with the same flow rate) to the glass cylinder through another Tenax tube (Tenax GR 60/80, Scientific Instrument Services, Old York, NJ, USA), which was inserted through a second opening, to clean the incoming air. The number of flowers inside the cylinder was counted to calculate volatile amounts per flower. All collections took place between 11^{00} and 15^{00}hrs. After scent collection the Tenax tubes were stored at -30˚C until chemical analysis.

For the analysis of floral volatiles, gas chromatography with mass selective detection (GC-MSD) was used. Samples were injected into a GC (Agilent 6890 N; Agilent Technologies, Santa Clara, CA, USA) using a Gerstel thermodesorption system (TDS3; Gerstel, Mühlheim, Germany) with cold injection (KAS4; Gerstel). The GC was equipped with a DB-5 column (0.32 mm ID, 0.25 lm film thickness, 30 m length), and helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Compound identification was done by comparing the spectra obtained from the natural samples with those of synthetic standard compounds. Standard compounds were also used for compound quantification using dose-response curves obtained for each volatile using characteristic ions (Schiestl et al. 2014). Because the two isomers of β-ocimene were strongly correlated to each other ($r = 0.97$, $P < 0.001$) we used their sum for statistical analysis. For the same reason the sum of the two aromatic

compounds p-anisaldehyde and 2-aminobenzaldehyde were used ($r = 0.61$, $P < 0.001$).

Petal length and width were measured in three fully opened flowers per individual (one petal per flower). Means of petal length and width were used to estimate the corolla size per flower as *π*length*width* (formula for an ellipse multiplied by 4 for the four petals).

RESULTS

Interaction between crab spider and pollinator attraction

In dual-choice behavioral assays testing the attractiveness of the three main floral scent compounds, crab spiders showed a significant preference for the floral monoterpene β-ocimene over an odorless control. In contrast, we detected no preference for the two aromatic compounds p-anisaldehyde and 2-aminobenzaldehyde (Figure 2A). Consistent with a preference for β-ocimene, crab spider presence on *B. laevigata* inflorescences in the Swiss lowland population was positively associated with the emission of β-ocimene (estimate ± s.e. = 0.04 ± 0.01, $z = 2.635$, $P = 0.008$) (Figure 2B). On average, plants visited by crab spiders emitted 60% more β-ocimene than plants where crab spiders were not observed. β-Ocimene also attracted bees: in dual-choice behavioral assays bees showed a significant preference for β-ocimene augmented plants compared to control plants (Figure 2C).

We used plot experiments to compare pollinator-mediated selection on floral traits in the presence and absence of crab spiders. While the selection on β-ocimene was affected by the presence of crab spiders, two floral traits were under significant selection independent of the treatment: number of flowers and the amount of aromatic scent compounds (Table 1). Both traits were associated positively with visits

by bees, for the number of flowers a selection gradient of 0.19 ± 0.03 (estimate ± s.e.) ($z = 6.547$, $P < 0.001$) was measured, for the aromatic scent compounds the selection gradient was 0.11 ± 0.03 (estimate ± s.e.) ($z = 3.759$, $P < 0.001$). However, the presence of crab spiders on 25-33% of the plants significantly affected selection for β-ocimene (trait x treatment effect) (Table 1). In the absence of crab spiders β-ocimene was under significant positive selection with a gradient of 0.12 ± 0.04 (estimate ± s.e.) ($z = 2.87$, $P = 0.004$). But when crab spiders were present, no selection on β-ocimene could be detected (estimate ± s.e. = -0.06 ± 0.04, $z = -1.59$, $P = 0.11$). Additionally, the time an inflorescence was occupied by a hunting crab spider had a significant negative effect on the number of bee visits (estimate ± s.e. = - 0.03 ± 0.01, $z = -3.015$, $P = 0.003$).

Table 1 Effect of floral traits and of the interactions between floral traits and crab spider presence on the attractiveness of flowers to bees (crab spiders hunting on 25-33% of the plants or no spiders present, N = 108 per treatment).

Trait	$\chi 2$	df	P value
β-Ocimene	0.643	1	0.42
Aromatics	14.727	1	**< 0.001**
Corolla size	0.005	1	0.94
Number of flowers	42.965	1	**< 0.001**
Spiders presence x β-ocimene	10.282	1	**0.001**
Spiders presence x aromatics	2.007	1	0.16
Spiders presence x corolla size	0.630	1	0.43
Spiders presence x number of flowers	0.846	1	0.36

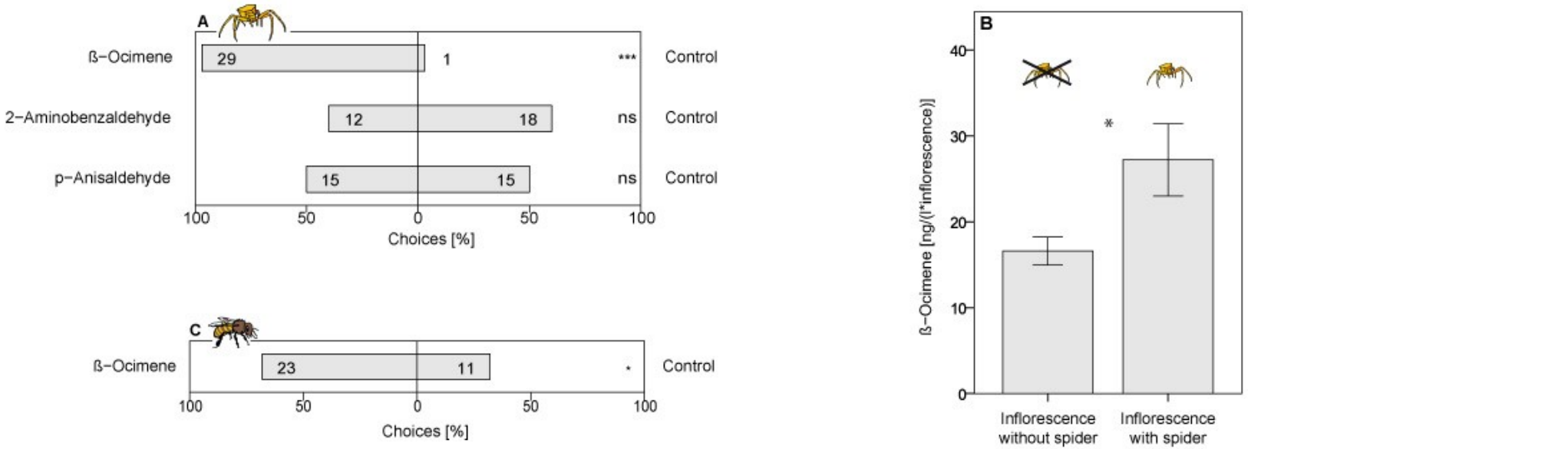


Figure 2 Preferences for floral scent compounds in crab spiders and bees. (*A*) Dual-choice behavioral assays testing the preference of crab spiders for the three main floral volatiles emitted by *Biscutella laevigata* against an odorless control. Numbers in bars are the absolute numbers of crab spiders selecting either side. Binomial test: ns: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001. (*B*) Emission of β-ocimene in plants with and without crab spiders hunting on inflorescence of *B. laevigata* in the Swiss lowland population. Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk. (*C*) Dual-choice behavioral assays testing the preference of bees for *B. laevigata* plants with augmented emission of β-ocimene against a control plant. Numbers in bars are the absolute number of landings on inflorescences. Binomial test: ns: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

Tritrophic interaction

In a plot experiment measuring the fitness effects of crab spiders and florivores separately and in combination, 89.9 % of the recorded captures by the crab spiders were florivores when they were present on inflorescences (Figure 1). Accordingly, the number of florivores was significantly reduced on plants with a crab spider on the inflorescence compared to plants without a spider (estimate ± s.e. = -1.4 ± 0.1, t = -10.11, P < 0.001) (Figure 3A). Total floral damage was also significantly lower for plants with spiders than for plants without spiders (estimate ± s.e. = -10.1 ± 1.7, t = -5.871, P < 0.001) (Figure 3B). While the presence of crab spiders on inflorescences did not affect plant fitness (estimate ± s.e. = -0.05 ± 0.04, t = -1.185, P = 0.58), florivores had a significant negative effect (estimate ± s.e. = -0.14 ± 0.04, t = -3.173, P = 0.022). Further, we found a significant interaction between spider and florivore presence on plant fitness; crab spiders reduced the negative effect of florivores on relative fitness (estimate ± s.e. = 0.14 ± 0.06, t = 2.205, P = 0.027) (Figure 3C). In the Swiss lowland population of *B. laevigata*, 12% of crab spider prey consisted of florivores in total.

After florivore infestation the emission of the main spider attractant β-ocimene significantly increased by 44 ± 16% (estimate ± s.e.) compared to control plants (t = 3.909, P < 0.001) (Figure 4A, see also Table S4 for inducibility of other compounds). Correspondingly, in dual-choice behavioral assays, crab spiders showed a significant preference for infested plants over control plants (Figure 4B). Also, in the natural plant population crab spiders occurred significantly more often on infested plants than expected by chance (P = 0.024). 69% of the plants that crab spiders had selected for hunting were infested with florivores although the infestation rate of the whole population was only 47%.

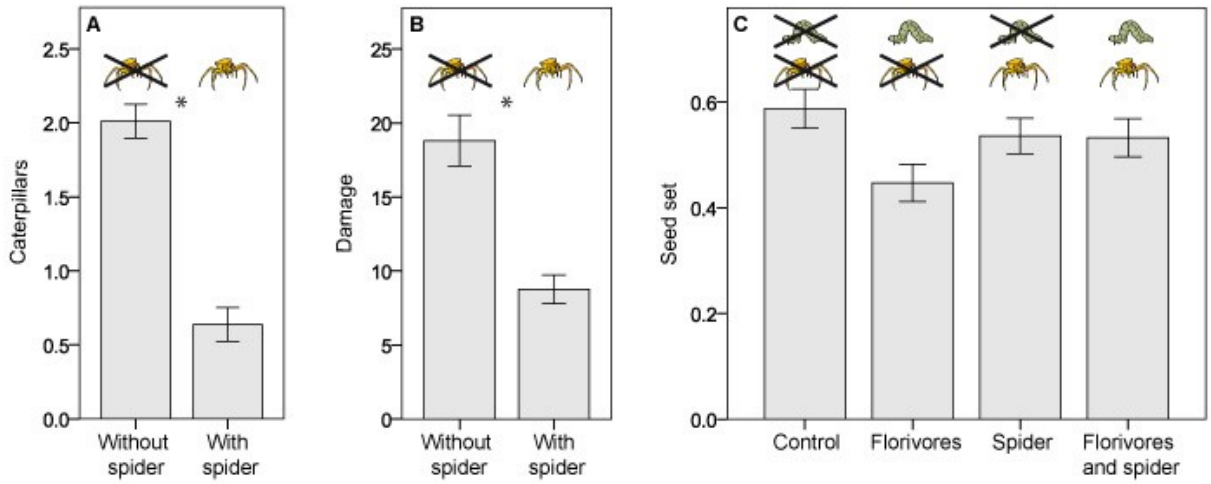


Figure 3 Effect of crab spiders on damage inflicted by florivory (*A*) Number of remaining florivores on plants with and without crab spiders on the inflorescence. Florivores were counted at the end of the day after 3 caterpillars have been placed on plants in the morning. Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk. (*B*) Florivore damage in plants with and without crab spiders. Damage was measured after *Plutella xylostella* feeding for four days; it was calculated as the sum of flowers and buds with feeding damage by florivores. Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk. (*C*) Fitness (measured as seed set) of *Biscutella laevigata* plants under four treatments: a) control (no crab spider or florivores); b) crab spider on the inflorescence; c) infestation with florivores; d) crab spider on the inflorescence and infestation with florivores. Crab spiders and florivores had a significant interactive effect on plant fitness; crab spiders reduced the negative effect of florivores (mixed effect model: estimate ± s.e. = 0.14 ± 0.06, t = 2.205, P = 0.027).

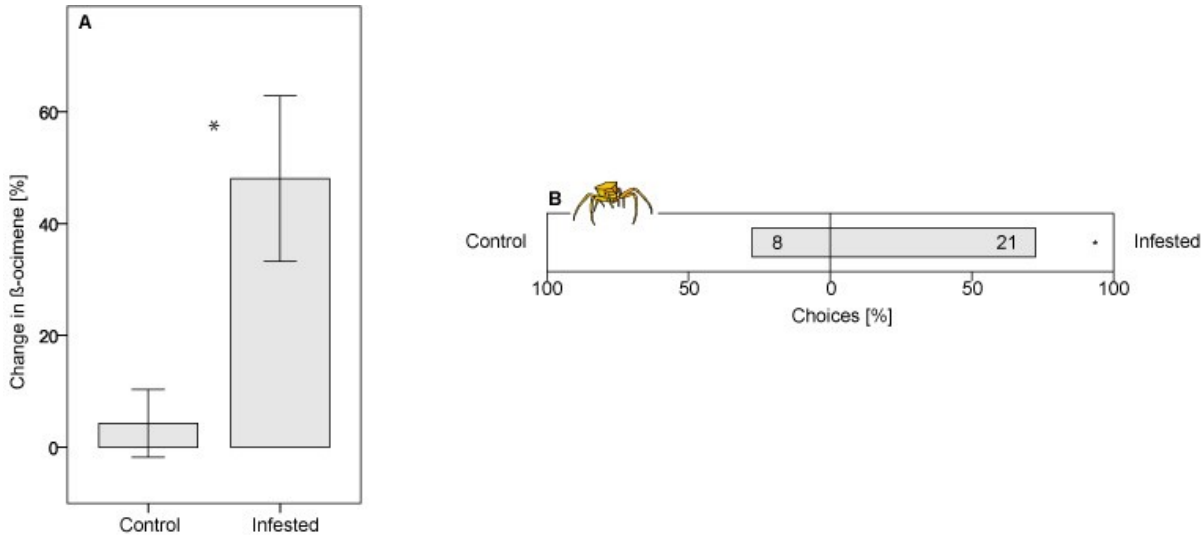


Figure 4 Tritrophic interaction between *Biscutella laevigata*, florivores and crab spiders. (*A*) Inducibility of β-ocimene in florivore-infested and control plants. Each bar represents a mean ± s.e., significant differences between treatments are indicated by an asterisk. (*B*) Dual-choice behavioral assays testing the preference of crab spiders for florivore infested *B. laevigata* plants against a control plants. Numbers in bars are the absolute number of choices to inflorescences. Binomial test: ns: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).

Local adaptation to crab spiders

We found a significant effect of altitude (low- vs highland) on the inducibility of β-ocimene emission (t = 2.034, P = 0.044) (Figure 5A). Inducibility was approximately double as high in lowland populations as in highland populations. In contrast, region (Swiss vs Italian) did not affect the inducibility of β-ocimene (t = 0.623, P = 0.53) (see also Table S4 for inducibility of other compounds). Further, before infestation with florivores, the absolute constitutive emission of β-ocimene did not differ between altitudes or regions (altitude: t = 1.682, P = 0.10; region: t = 0.132, P = 0.90). After infestation, however, lowland populations emitted significantly higher amounts than highland populations (estimate ± s.e. = 1.7 ± 0.7, t = 2.444, P = 0.016), while region still did not affect β-ocimene emission (t = 0.545, P = 0.59) (Figure 5B).

In dual choice behavioral assays crab spiders showed no preference for lowland over highland plants in the absence of florivores, neither in the Swiss nor the Italian lineage. After florivore infestation, however, crab spiders significantly preferred lowland over highland plants in both regions (Figure 5C).

DISCUSSION

Complex webs of plant-animal interactions can give rise to conflicting and nonadditive selection, with consequences for the evolution of floral signals. The relevance of indirect plant-animal interactions for flower evolution is little known, and virtually unexplored for plants and crab spiders, despite their common occurrence on flowers. Here we show nonadditive effects of crab spiders and florivores on plant fitness, with spiders having a net positive effect when removing florivores. We also show that plants are locally adapted to attracting crab spiders after herbivore attack though induced floral volatile emission. Our results demonstrate the relevance of crab spiders in driving floral trait evolution in *B. laevigata* and give insights into patterns of selection that individual components and whole plant-animal interactions webs impose on floral traits.

As many crab spiders hunt specifically at flowers, their effects on plant fitness has so far mostly been considered in connection to reducing pollinator visits (Dukas 2001; but see Gonzalez et al. 2009). Signals attracting pollinators are especially relevant for crab spiders as pollinators form a considerable proportion of their diet. Our finding of β-ocimene as the attractive signal for both pollinators and crab spiders suggest that the crab spiders' preference has evolved to exploit the established communication channel between plants and pollinators forcing a conflict on plants between the attraction of pollinators and the avoidance of antagonists. Similar to our results, in *Chrysanthemum frutescens* flowers, the crab spider *Thomisus spectabilis*

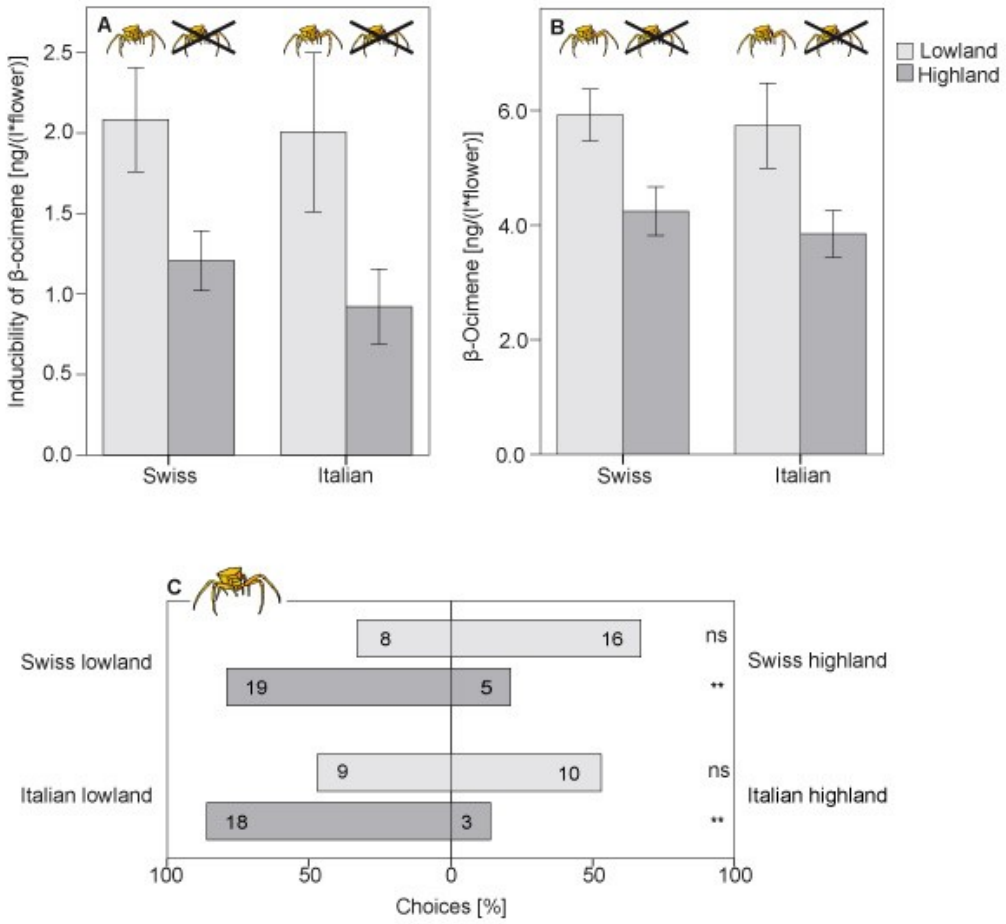


Figure 5 Adaptation of *Biscutella laevigata* populations to crab spiders. (*A*) Inducibility of β-ocimene emission in lowland (with spiders) and highland (without spiders) populations, in the Swiss and Italian lineage. Inducibility was significantly higher in lowland populations than in highland populations (linear model: estimate ± 0.09 ± 0.04, t = 2.034, P = 0.044). The lineage (Swiss vs Italian) on the other hand did not affect the inducibility of this compound (t = 0.565, P = 0.57). (*B*) Absolute emission of β-ocimene after florivore infestation in low- and highland populations, in the Swiss and Italian lineage. Lowland populations emitted significantly higher amounts of β-ocimene compared to highland populations (estimate ± s.e. = 1.7 ± 0.7, t = 2.444, P = 0.016). The lineage (Swiss vs Italian) on the other hand had no effect on the amount of β-ocimene (t = 0.545, P = 0.59). (*C*) Dual-choice behavioral assays testing the preference of crab spiders for lowland *B. laevigata* plants against highland plants, for the Swiss and Italian lineage separately. Dark grey: infested plants; light grey: control plants. Numbers in bars are the absolute number of crab spiders selecting each side. Binomial test: ns: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

and honey bees overlap in their preferences for floral symmetry as well as floral scent (although the chemical identity of the attractive scent was not identified in that system) (Heiling et al. 2004; Wignall et al. 2006). Such exploitative preferences should be under strong selection to maximize prey encounter in “sit-and-wait” predators as this strategy is only effective when sufficient numbers of prey pass by within striking distance of predators. A preference for β-ocimene might be especially successful as this floral volatile is emitted by many plant species and attracts various pollinator taxa (Knudsen et al. 1993; Takabayashi et al. 1994; Byers et al. 2014; Dotterl et al. 2012).

Various plant traits have evolved to reduce the negative effect of herbivory on plant fitness including indirect defense (Heil 2008; Rosenthal and Kotanen 1994; Hanley et al. 2007; Howe and Jander 2008; Strauss and Agrawal 1999). In *B. laevigata* crab spiders are detrimental for plant fitness in the absence of florivores, but they become beneficial when florivores are present. This suggests that plants are under selection to attract spiders only when attacked by florivores, which we confirmed by showing higher attractiveness of infested plant for spiders, likely mediated by induced floral β-ocimene emission. β-Ocimene is one of the most common herbivore-induced plant volatiles and is involved in tritrophic interactions with many different types of herbivores (Takabayashi et al. 1994; Magalhaes et al. 2012; Suckling et al. 2012; Han and Chen 2002; Zhang et al. 2013; Dicke et al. 1990; Agrawal et al. 2002; Kessler and Baldwin 2001). The situative beneficial effect of the crab spider *T. onustus* on plant fitness might thus occur in several plant species they visit for hunting.

Several studies have demonstrated local adaptation in plants to the presence or abundance of herbivores through direct defense (Kalske et al. 2012; Garrido et al. 2012; Sork et al. 1993; Arany et al. 2009; Muola et al. 2010). In contrast, local

adaptation of plant populations to indirect defense across trophic levels has not yet been documented. Our study provides evidence for local adaptation of *B. laevigata* populations to the tritrophic interaction between plant, florivores and the crab spider *T. onustus* through induced β-ocimene emission. The model by Higginson et al. (2010) predicted the evolution of predator-attracting traits under conditions which positively influence the net effect of crab spiders on plant fitness: high pollinator abundance and –effectiveness, and strong florivory. These conditions are met in our *B. laevigata* populations, where florivore infestation reached up to 40% and decreased fitness by 45% in infested plants (see SI Appendix). Also, buckler mustard populations with crab spiders are unlikely pollinator limited as pollinators are usually abundant, and the low number of ovule per flower (*Biscutella* species have fruits with two seeds;) makes few pollen grains sufficient for full seed set (see SI Appendix for pollen limitation and Table S2 pollinator effectiveness). Finally, the proportion of florivores in the prey of crab spiders was 12% in the Swiss lowland population indicating an occasional positive effect by predators on plant fitness.

In conclusion, our data suggest a common, so far overlooked role of crab spiders in floral trait evolution. Compared to other natural enemies, crab spiders should impose strong selection as they can feed several florivores per day and - unlike parasitoids - kill their prey immediately. Also, crab spiders occur worldwide with over 2000 species and are commonly found on flowers (Morse 1981; Rocha and Rinaldi 2011; De Souza and Martins 2004). Thus, their impact on plant evolution may be widespread among angiosperms.

ACKNOWLEDGEMENTS

We would like to thank Rayko Jonas and Markus Meierhofer for their help with plant cultivation and Christian Parisod for his advice on the cultivation conditions. Also, we

thank Alice Balmer and Tanja Christoffel for their help with the experiments and Franz Huber for his support in the GC lab. Oliver Kindler (Syngenta AG) kindly provided the *Plutella* larvae used in this study. The research leading to these results has received funding from the European Union's Seventh Framework Program ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement no 281093.

REFERENCES

Agrawal AA, Janssen A, Bruin J, Posthumus MA, Sabelis MW (2002) An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecology Letters 5 (3):377-385. doi:10.1046/j.1461-0248.2002.00325.x

Antiqueira PAP, Romero GQ (2016) Floral asymmetry and predation risk modify pollinator behavior, but only predation risk decreases plant fitness. Oecologia 181 (2):475-485. doi:10.1007/s00442-016-3564-y

Arany AM, de Jong TJ, van der Meijden E (2009) Herbivory and local genetic differentiation in natural populations of Arabidopsis thaliana (Brassicaceae). Plant Ecology 201 (2):651-659. doi:10.1007/s11258-008-9530-y

Armbruster WS (1997) Exaptations link evolution of plant-herbivore and plant-pollinator interactions: A phylogenetic inquiry. Ecology 78 (6):1661-1672

Brody AK, Mitchell RJ (1997) Effects of experimental manipulation of inflorescence size on pollination and pre-dispersal seed predation in the hummingbird-pollinated plant Ipomopsis aggregata. Oecologia 110 (1):86-93. doi:10.1007/s004420050136

Bronstein JL, Alarcon R, Geber M (2006) The evolution of plant-insect mutualisms. New Phytologist 172 (3):412-428. doi:10.1111/j.1469-8137.2006.01864.x

Byers K, Bradshaw HD, Riffell JA (2014) Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (Mimulus). Journal of Experimental Biology 217 (4):614-623. doi:10.1242/jeb.092213

De Souza ALT, Martins RP (2004) Distribution of plant-dwelling spiders: Inflorescences versus vegetative branches. Austral Ecology 29 (3):342-349

Dicke M, Vanbeek TA, Posthumus MA, Bendom N, Vanbokhoven H, Degroot AE (1990) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions - involvement of host plants in its production. Journal of Chemical Ecology 16 (2):381-396. doi:10.1007/bf01021772

Dotterl S, Jahreiss K, Jhumur US, Jurgens A (2012) Temporal variation of flower scent in Silene otites (Caryophyllaceae): a species with a mixed pollination system. Botanical Journal of the Linnean Society 169 (3):447-460. doi:10.1111/j.1095-8339.2012.01239.x

Dukas R (2001) Effects of predation risk on pollinators and plants. In: Chittka L, Thomson, J.D. (ed) Cognitive Ecology of pollination. Cambridge University Press, Cambridge, pp 214-236

Garrido E, Andraca-Gomez G, Fornoni J (2012) Local adaptation: simultaneously considering herbivores and their host plants. New Phytologist 193 (2):445-453. doi:10.1111/j.1469-8137.2011.03923.x

Gomez JM (2003) Herbivory reduces the strength of pollinator-mediated selection in the Mediterranean herb Erysimum mediohispanicum: Consequences for plant specialization. American Naturalist 162 (2):242-256

Gomez JM (2005) Non-additive effects of herbivores and pollinators on Erysimum mediohispanicum (Cruciferae) fitness. Oecologia 143 (3):412-418. doi:10.1007/s00442-004-1809-7

Gómez JM (2008) Sequential conflicting selection due to multispecific interactions triggers evolutionary trade-offs in a monocarpic herb. Evolution 62 (3):668-679. doi:10.1111/j.1558-5646.2007.00312.x

Goncalves-Souza T, Omena PM, Souza JC, Romero GQ (2008) Trait-mediated effects on flowers: Artificial spiders deceive pollinators and decrease plant fitness. Ecology 89 (9):2407-2413. doi:10.1890/07-1881.1

Gonzalez A, Liljesthrom G, Minervino E, Castro D, Gonzalez S, Armendano A (2009) Predation by Misumenops pallidus (Araneae: Thomisidae) on insect pests of soybean cultures in Buenos Aires Province, Argentina. Journal of Arachnology 37 (3):282-286

Gross K, Sun M, Schiestl FP (2016) Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. Plos One 11 (2):e0147975-e0147975. doi:10.1371/journal.pone.0147975

Han BY, Chen ZM (2002) Composition of the volatiles from intact and mechanically pierced tea aphid-tea shoot complexes and their attraction to natural enemies of the tea aphid. Journal of Agricultural and Food Chemistry 50 (9):2571-2575. doi:10.1021/jf010681x

Hanley ME, Lamont BB, Fairbanks MM, Rafferty CM (2007) Plant structural traits and their role in anti-herbivore defence. Perspectives in Plant Ecology Evolution and Systematics 8 (4):157-178. doi:10.1016/j.ppees.2007.01.001

Heil M (2008) Indirect defence via tritrophic interactions. New Phytologist 178 (1):41-61. doi:10.1111/j.1469-8137.2007.02330.x

Heiling AM, Cheng K, Herberstein ME (2004) Exploitation of floral signals by crab spiders (Thomisus spectabilis, Thomisidae). Behavioral Ecology 15 (2):321-326. doi:10.1093/beheco/arh012

Heiling AM, Chittka L, Cheng K, Herberstein ME (2005) Colouration in crab spiders: substrate choice and prey attraction. Journal of Experimental Biology 208 (10):1785-1792. doi:10.1242/jeb.01585

Higginson AD, Ruxton GD, Skelhorn J (2010) The impact of flower-dwelling predators on host plant reproductive success. Oecologia 164 (2):411-421. doi:10.1007/s00442-010-1681-6

Hoeksema JD, Bruna EM (2015) Context-dependent outcomes of mutualistic interactions. In: Bronstein JL (ed) Mutualism. Oxford University Press, Oxford, pp 181-202

Howe GA, Jander G (2008) Plant immunity to insect herbivores. In: Annual Review of Plant Biology, vol 59. Annual Review of Plant Biology. pp 41-66. doi:10.1146/annurev.arplant.59.032607.092825

Kalske A, Muola A, Laukkanen L, Mutikainen P, Leimu R (2012) Variation and constraints of local adaptation of a long-lived plant, its pollinators and specialist herbivores. Journal of Ecology 100 (6):1359-1372. doi:10.1111/j.1365-2745.2012.02008.x

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291 (5511):2141-2144. doi:10.1126/science.291.5511.2141

Knudsen JT, Tollsten L, Bergstrom LG (1993) Floral scents - A checklist of volatile compounds isolated by headspace techniques. Phytochemistry 33 (2):253-280. doi:10.1016/0031-9422(93)85502-i

Levy G (1970) Life cycle of *Thomisus onustus* (Thomsidae - Araneae) and outlines for classification of life histories of spiders Journal of Zoology 160:523-&

Magalhaes DM, Borges M, Laumann RA, Sujii ER, Mayon P, Caulfield JC, Midega CAO, Khan ZR, Pickett JA, Birkett MA, Blassioli-Moraes MC (2012) Semiochemicals from Herbivory Induced Cotton Plants Enhance the Foraging Behavior of the Cotton Boll Weevil, Anthonomus grandis. Journal of Chemical Ecology 38 (12):1528-1538. doi:10.1007/s10886-012-0216-5

Morse DH (1981) PREY CAPTURE BY THE CRAB SPIDER MISUMENA-VATIA (CLERCK) (THOMISIDAE) ON 3 COMMON NATIVE FLOWERS. American Midland Naturalist 105 (2):358-367. doi:10.2307/2424754

Muola A, Mutikainen P, Lilley M, Laukkanen L, Salminen JP, Leimu R (2010) Associations of plant fitness, leaf chemistry, and damage suggest selection mosaic in plant-herbivore interactions. Ecology 91 (9):2650-2659. doi:10.1890/09-0589.1

Olowokudejo JD, Heywood VH (1984) Cyto-taxonomy and breeding system of the genus *Biscutella* (Cruciferae) Plant Systematics and Evolution 145 (3-4):291-309. doi:10.1007/bf00983955

Parisod C, Besnard G (2007) Glacial in situ survival in the Western Alps and polytopic autopolyploidy in Biscutella laevigata L. (Brassicaceae). Molecular Ecology 16 (13):2755-2767. doi:10.1111/j.1365-294X.2007.03315.x

Reed DW, Pivnick KA, Underhill EW (1989) Identification of chemical oviposition stimulants for the diamondback moth, *Plutella xylostella*, present in 3 species of Brassicaceae Entomologia Experimentalis Et Applicata 53 (3):277-286

Rey PJ, Herrera CM, Guitian J, Cerda X, Sanchez-Lafuente AM, Medrano M, Garrido JL (2006) The geographic mosaic in predispersal interactions and selection on Helleborus foetidus (Ranunculaceae). Journal of Evolutionary Biology 19 (1):21-34

Roberts MJ (1996) Spiders - Britain and Northern Europe.

Rocha LC, Rinaldi IMP (2011) Crab spiders (Araneae: Thomisidae) in flowering plants in a Brazilian "Cerrado" ecosystem. Brazilian Journal of Biology 71 (2):359-364

Romero GQ, Vasconcellos-Neto J (2004) Beneficial effects of flower-dwelling predators on their host plant. Ecology 85 (2):446-457. doi:10.1890/02-0327

Rosenthal JP, Kotanen PM (1994) Terrestrial plant tolerance to herbivory Trends in Ecology & Evolution 9 (4):145-148. doi:10.1016/0169-5347(94)90180-5
Schaefer MH, Ruxton GD (2011) Plant-animal communication. New York, Oxford university press
Schiestl FP, Huber FK, Gómez JM (2011) Phenotypic selection on floral scent: trade-off between attraction and deterrence? Evolutionary Ecology 25 (2):237-248. doi:10.1007/s10682-010-9409-y
Schiestl FP, Johnson SD (2013) Pollinator-mediated evolution of floral signals. Trends in Ecology & Evolution 28 (5):307-315. doi:10.1016/j.tree.2013.01.019
Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. New Phytologist 203 (1):257-266
Sork VL, Stowe KA, Hochwender C (1993) Evidence for local adaptation in closely adjacent subpopulations of northern red oak (*Quercus rubra L*) expressed as resistance to leaf herbivores. American Naturalist 142 (6):928-936. doi:10.1086/285581
Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. Trends in Ecology & Evolution 14 (5):179-185. doi:10.1016/s0169-5347(98)01576-6
Strauss SY, Irwin RE (2004) Ecological and evolutionary consequences of multispecies plant-animal interactions. Annual Review of Ecology Evolution and Systematics 35:435-466. doi:10.1146/annurev.ecolsys.35.112202.130215
Suckling DM, Twidle AM, Gibb AR, Manning LM, Mitchell VJ, Sullivan TES, Wee SL, El-Sayed AM (2012) Volatiles from Apple Trees Infested with Light Brown Apple Moth Larvae Attract the Parasitoid Dolichogenidia tasmanica. Journal of Agricultural and Food Chemistry 60 (38):9562-9566. doi:10.1021/jf302874g
Sun M, Gross K, Schiestl FP (2014) Floral adaptation to local pollinator guilds in a terrestrial orchid. Annals of Botany 113 (2):289-300. doi:10.1093/aob/mct219
Takabayashi J, Dicke M, Posthumus MA (1994) Volatile herbivore-induced terpenoids in plant mite interactions - variation caused by biotic and abiotic factors. Journal of Chemical Ecology 20 (6):1329-1354. doi:10.1007/bf02059811
Theis N, Adler LS (2012) Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. Ecology 93 (2):430-435
Thompson JN, Cunningham BM (2002) Geographic structure and dynamics of coevolutionary selection. Nature 417 (6890):735-738. doi:10.1038/nature00810
Thompson JN, Schwind C, Guimaraes PR, Jr., Friberg M (2013) Diversification through multitrait evolution in a coevolving interaction. Proceedings of the National Academy of Sciences of the United States of America 110 (28):11487-11492. doi:10.1073/pnas.1307451110
van der Niet T, Johnson SD (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. Trends in Ecology & Evolution 27 (6):353-361. doi:10.1016/j.tree.2012.02.002
Wignall AE, Heiling AM, Cheng K, Herberstein ME (2006) Flower symmetry preferences in honeybees and their crab spider predators. Ethology 112 (5):510-518. doi:10.1111/j.1439-0310.2006.01199.x
Zhang PJ, Xu CX, Zhang JM, Lu YB, Wei JN, Liu YQ, David A, Boland W, Turlings TCJ (2013) Phloem-feeding whiteflies can fool their host plants, but not their parasitoids. Functional Ecology 27 (6):1304-1312. doi:10.1111/1365-2435.12132

SUPPORTING INFORMATION

Pollinator guild

To characterize the pollinator guilds of the Swiss lowland and highland population, floral visitors were surveyed during the flowering time during 9 and 7 days respectively. Observations of floral visitors were made by random walking across field sites for at least one hour per day whereas all observed visits were recorded to measure relative pollinator abundances. To identify pollinators, we captured some individuals and subsequently identified them at the genus or family level (Chinery, 2004). Additionally we measured the pollination effectiveness by quantifying the pollen carryover and the number of visited flowers per visit. Pollination effectiveness was assessed for the orders Hymenoptera and Diptera which included the most abundant visitors. To measure pollen carryover, we randomly chose 10 plants per population and bagged inflorescences in bud stage. After flower opening, the bags were removed and all opened flowers were emasculated to avoid selfing. After single visits by floral visitors we counted the number of pollen grains on the stigmas of visited flowers. Pollen counts were done under a stereoscopic binocular microscope (Nikon Nature Scope, Nikon, Japan). Pollen carryover was measured for 13 flower visitors in the lowlands and 25 in the highlands (some of the bagged plants were used to record more than one visit). Further, the number of visited flowers per single visit was recorded for 54 pollinators in the lowland population and 83 in the highland population.

In total, we observed 23 and 17 different genera of floral visitors in the lowland and highland population respectively. While Hymenopterans were the most abundant pollinators in the lowlands Dipterans and one sawfly species belonging to the genus Tenthredo were the most abundant pollinators in the highlands (Table S1). Further,

pollen carryover and the number of visited flowers per visit for Hymenopterans and Dipterans are given Table S2.

Table S2: Mean (± s.e.) pollen carryofer and number of visited flowers for insect visitors from the order Hymenoptera and Diptera in the Swiss lowland and highland populations.

Population	Insect taxa	Pollen carryover	Number of visited flowers
Lowland	Hymenoptera	10.6 ± 1.4	8.3 ± 1.4
	Diptera	6.9 ± 2.0	4.9 ± 0.7
Highland	Hymenoptera	14.0 ± 1.3	3.7 ± 0.7
	Diptera	5.4 ± 0.5	5.1 ± 0.4

Spider abundance

The spider abundance of the Swiss lowland population was measured by scanning around 100 plants for their association with a crab spider. In total spider abundance was measured three times, once in 2014 and twice in 2016.

While in 2014 25% of the recorded plants had a crab spider on the inflorescence in 2016 the ratios were 26% and 28% respectively.

Scent solutions for septa preparation

To obtain emission rates from septa similar to those of *B. laevigata* plants, they were soaked in solutions of the pure compounds in dichloromethane. We used solutions of 7 µl ml^{-1} β-ocimene (mixture of isomeres, ≥ 90%, Sigma Aldrich, St. Louis, USA), 7 µl ml^{-1} 2-aminobenzaldehyde (≥ 98%, Sigma Aldrich, St. Louis, USA) and 2 µl ml^{-1} p-anisaldehyde (≥ 98%, Sigma Aldrich, St. Louis, USA). Emission rates from septa in comparison to actual plants are give in Table S3.

Table S1: Relative abundance of pollinator guilds for the Swiss lowland and highland populations. Relative abundances for whole insect orders are given in bold.

Insect taxa		Relative abundance	
Order	Family	Lowland	Highland
Hymenoptera	Apidae	0.272	0.016
	Halictidae	0.135	-
	Xyelidae	0.007	-
	Megalodontidae	0.004	-
	Tenthredinidae	0.029	0.271
	Formicidae	-	0.062
	Total	**0.446**	**0.348**
Diptera	Syrphidae	0.219	0.135
	Platypezidae	-	0.163
	Scatophagidae	-	0.186
	Total	**0.219**	**0.485**
Lepidoptera	Lycaenidae	0.087	0.007
	Nymphalidae	0.047	0.005
	Pieridae	0.004	0.009
	Torticidae	0.029	0.019
	Noctuidae	0.004	-
	Total	**0.171**	**0.040**
Coleoptera	Cerambycidae	0.007	0.107
	Scarabaeidae	0.004	-
	Cantharidae	0.004	-
	Elateridae	-	0.005
	Total	**0.015**	**0.112**
Hemiptera	Pentatomidae	**0.131**	**0.014**

Table S3: Amounts (pg l^{-1}) of the three main volatile compounds of *B. laevigata* emitted by plants and septa. Scent was collected from 95 plants in the Swiss lowland population and 5 septa as described in the main manuscript.

Compound	Plant		Septa
	mean ± s.e.	maximum	mean ± s.e.
β-Ocimene	20.0 ± 2.0	91.0	39.8 ± 1.6
2-Aminobenzaldehyde	24.8 ± 2.6	106.7	46.5 ± 1.0
p-Anisaldehyde	26.4 ± 2.4	153.4	39.6 ± 0.7

Table S4: Mean (± s.e.) amounts (pg l^{-1} $flower^{-1}$) of the three main compounds of *B. laevigata* before infestation with florivores (constitutive emission) and after infestation. Additionally the induced amount was calculated as the change in emission through florivory (N = 31 for Swiss lowland population, N = 30 for Swiss highland population, N = 24 for Italian lowland population, N = 26 for Italian highland population).

Compound	Constitutive			
	Swiss		Italian	
	Lowland	Highland	Lowland	Highland
β-Ocimene	3.8 ± 0.4	3.1 ± 0.3	3.7 ± 0.4	3.0 ± 0.3
2-Aminobenzaldehyde	9.9 ± 0.7	3.7 ± 0.5	8.7 ± 1.4	4.1 ± 0.8
p-Anisaldehyde	5.4 ± 0.4	0.7 ± 0.3	0.2 ± 1.4	0.8 ± 0.2
	Infested			
	Swiss		Italian	
	Lowland	Highland	Lowland	Highland
β-Ocimene	5.9 ± 0.4	4.2 ± 0.4	5.7 ± 0.7	3.9 ± 0.4
2-Aminobenzaldehyde	9.9 ± 1.0	1.9 ± 0.4	4.0 ± 0.2	1.4 ± 0.4
p-Anisaldehyde	4.1 ± 0.5	0.4 ± 0.07	0.7 ± 0.03	0.3 ± 0.06
	Induced (infested - constitutive)			
	Swiss		Italian	
	Lowland	Highland	Lowland	Highland
β-Ocimene	2.1 ± 0.3	1.2 ± 0.2	2.0 ± 0.5	0.9 ± 0.2
2-Aminobenzaldehyde	0.005 ± 0.9	-1.9 ± 0.3	-4.5 ± 1.2	-2.8 ± 0.5
p-Anisaldehyde	-1.3 ± 0.3	-0.4 ± 0.09	-0.03 ± 0.04	-0.5 ± 0.1

Florivory

Florivore infestation rates were measured for the Swiss low- and highland populations in the years 2016 and 2014 respectively. We scanned 105 individuals in the lowlands and 56 individuals in the highlands for the presence of florivores on inflorescences. Although different beetle species occasionally feed on *B. laevigate* petals, we only recorded larvae (mainly caterpillars and beetle larvae) that feed on whole flowers and can drastically reduce plant fitness. Additionally we measured plant fitness for all scanned individuals in the highland population after seed development. To control for differences in flower number between individuals fitness was measured as relative seed set. As *B. laevigata* can develop 2 seeds per flower

maximally, the relative seed set was calculated as *(number of seeds)/(2*number of flowers)*. To test for an effect of florivory on plant fitness we conducted a t-test between infested and non-infested plants.

Infestation rates were 43% in the Swiss lowlands and 36% in the highlands respectively. Further, florivory significantly reduced plant fitness by 45 % on average ($t = 2.2357$, $P = 0.031$).

Pollen limitation

To get an estimate of the degree of pollen limitation in the Swiss lowland population of *B. laevigata*, we measured the mean female fitness of the population and of some plants receiving supplemental hand-pollination. The mean fitness of the population was determined by measuring the relative seed set in 76 plants after the flowering period in 2014. Supplemental hand-pollination was done in an additional 10 plants for 3 flowers per individual. As *B. laevigata* can develop 2 seeds per flower maximally, the fitness was calculated as *(number of seeds)/(2*number of flowers)*.

The mean fitness of the population was 0.60 ± 0.03. Supplemental hand-pollination increased the fitness to 0.78 ± 0.10.

FINAL REMARKS

Specific plant-animal interactions can be affected by the context in which they take place. First, an animal's behavior and preferences for floral traits can be affected by the environment and the previous experiences the animal made. Furthermore, the fitness outcome of a specific interaction can depend on the presence of other interactions or the correlation between plant traits. Such context-dependence can cause complex selection patterns and affect the evolution of plant traits. The purpose of this thesis was the contribution to the understanding of these interdependencies in plant-animal communication and floral trait evolution. In the first part of the thesis (*chapter I and II*) we focused on the role of signal-reward correlations in plant-pollinator communication and its consequences for flower evolution. In the second part (*chapter III and IV*) we investigated the interplay between different plant-animal interactions (including pollinators, herbivores and predators) and their interactive effects on plant fitness and selection.

The role of a specific floral signal in pollinator attraction can depend strongly on the situation that pollinators experience. In our study bumble bees developed preferences for floral signals only when these were associated with reward amounts (honest signals). Further, in *Brassica rapa,* bumble bees showed a change in preferences for floral traits in the presence of cabbage butterflies. Additionally, in *Biscutella laevigata*, bees avoided plants with crab spiders on inflorescences even when these emitted attractive signals. Our results therefore demonstrate that plant-animal communication can depend on the interplay between different plant traits but also the presence of other animals interacting with the plant.

We further showed that the selection on plant traits imposed by specific plant-animal interactions can depend on the presence of other interacting animals. In both Brassicaceae species under study plants experienced a conflict between the attraction of mutualists and the avoidance of antagonists which caused conflicting selection in the presence of both interactions. Further, in both species we found

nonadditive fitness effects by different interactions. While in *B. laevigata*, the effect of a generalist predator on plant fitness depended on the plant's infestation with florivores, in *B. rapa* florivore infestation strongly reduced the positive fitness effect of pollination, weakening pollinator-mediated selection.

Additionally we showed that plant-animal interactions can impose selection on the association between different plant traits. The pollinating herbivore *Pieris brassicae* selected against trait combinations causing high herbivory in favour of combinations that avoided herbivores but still attracted a more efficient pollinator. Also, pollinator-mediated selection on floral signals depended on the association between floral signals and reward (honest signalling). The maintenance of this association was demonstrated to depend on the pollinators' learning capacities and behavior as well as metabolic constraints in the plant. Low rewarding plants are prevented from cheating by high costs of signal and seed production. The selection on this trait association should thus depend on various environmental factors as resource limitation and the composition of the pollinator guild. It would be interesting to further explore the role of these factors in maintaining mutualistic plant-animal interactions by the use of an experimental evolution approach and mathematical modelling in future studies.

Overall, our study gave insights into the complexity of plant-animal interactions and clearly demonstrates that floral evolution can be affected by the interplay between different biotic interactions and floral trait associations. Additionally, in our study the expression of certain floral signals as well as the presence of certain plant-animal interactions were influenced by abiotic factors. For certain plant species the selection on floral traits may thus strongly vary depending on the location and year of measurement. Further, global change including biodiversity loss, climate change and nutrient accumulation in soils can be expected to strongly affect the geographic pattern and composition of plant-animal interactions as well as physiological constraints in plants. The results of our study may thus help to predict plant evolution and the stability of mutualistic interactions.

ACKNOWLEDGEMENTS

First, I would like to thank my Ph.D. supervisor Prof. Dr. Florian Schiestl for his guidance, trust and the liberty he gave me in pursuing my Ph.D.. Already during my bachelor studies he strongly contributed to my arousing interest in plant-animal interactions and evolutionary biology. With his open mind and the critical questions he asked he further supported my understanding of these topics during my Ph.D.

Further I would like to thank the members of my Ph.D. committee Prof. Dr. Marta Manser and Dr. Christian Parisod for their personal support and the good discussions we had.

This Ph.D. was kindly funded by grants from the European Union's Seventh Framework Program (FP7/2007-2013, FP7/2007-2011) under grant agreement no. 281093.

Special Thanks go to all the people who supported me with data collection and analysis. Ed Connor and Franz Huber introduced me into the collection and analysis of floral scent. Alice Balmer supported me strongly in the collection of data in the greenhouse and field. Beatrice Christoffel reared the experimental insects. Markus Meierhofer and Rayko Jonas took care of the hundreds of plants I used for these studies. Karin Gross introduced me into the use of flow cytometry.

I would also like to thank all the members of the Schiestl group for the many interesting and stimulating discussions we had about evolutionaly biology - Daniel Gervasi, Roman Kellenberger, Sergio Ramos Castro, Pengjuan Zu, Karin Gross,

Mimi Sun, Judith Trunschke, Martin von Arx, Alok Gupta, Jörg Vogt, Philipp Schluter, Kelsey Byers, Laura Pineiro and Srignanakshi Iyer.

Many thanks go also to all my colleagues at the Institute of Systematic and Evolutionary Botany with whom I had a great time during my Ph.D..

I additionally would like to thank my partner Florian Olomski and my friends Deborah Admaty, Lea Bona, Irene Kobler and Biho Song for their mental support during all the times that my experiments did not proceeded as expected. I am also thankful for the home I had found in the Albi-WG and to the diverse people I lived with and who kept my mind open and interested in topics outside biology.

Most of all, I would like to thank my parents Urs and Ursi Knauer, my brother Tobias Knauer and my godmother Andrea Fahrländer for their lifelong company and guidance. They always supported my ambitions and interests and significantly shaped my values as critical thinking, sense of justice and independence.

CURRICULUM VITAE

2013 – present Ph.D. in Evolutionary Biology

Institute of Systematic and Evolutionary Botany, University of Zurich.

Topic: “The Effect of Pollinators, Herbivores and Predators on Floral Trait Evolution”.

2011-2013 M.Sc. in Systematics and Evolution.

Institute of Systematic and Evolutionary Botany, University of Zurich.

Topic: “Mechanisms of Pollinator Attraction in *Brassica rapa* flowers”

2007-2011 B.Sc. in Biology

University of Zurich